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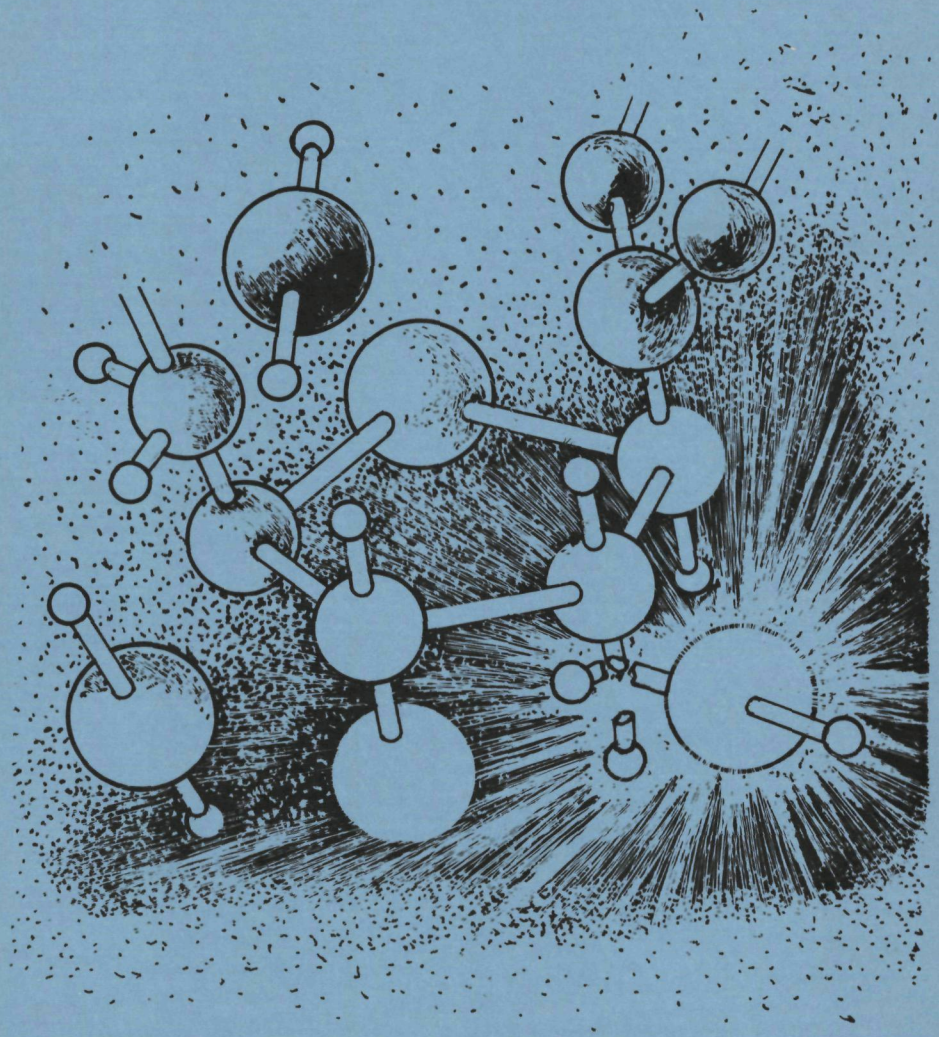
# MUTAGENICITY OF GAMMA IRRADIATED NUCLEIC ACID CONSTITUENTS



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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE WISKUNDE EN NATUURWETENSCHAPPEN AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN, OP GEZAG VAN DE RECTOR MAGNIFICUS PROF. DR. P. G. A. B. WIJDEVELD VOLGENS BESLUIT VAN HET COLLEGE VAN DECANEN IN HET OPENBAAR TE VERDEDIGEN OP VRIJDAG 26 SEPTEMBER 1980 DES NAMIDDAGS TE 2 00 UUR PRECIES

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JOHANNES WILHELMUS GERARDUS MARIA WILMER

geboren te DELFT

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The investigations have been carried out at the Institute for Atomic Sciences in Agriculture (Foundation ITAL) in Wageningen and, partly, at the Department of Radiation Genetics and Chemical Mutagenesis, University of Leiden.

*Aan mijn ouders*
*Aan Désirée*

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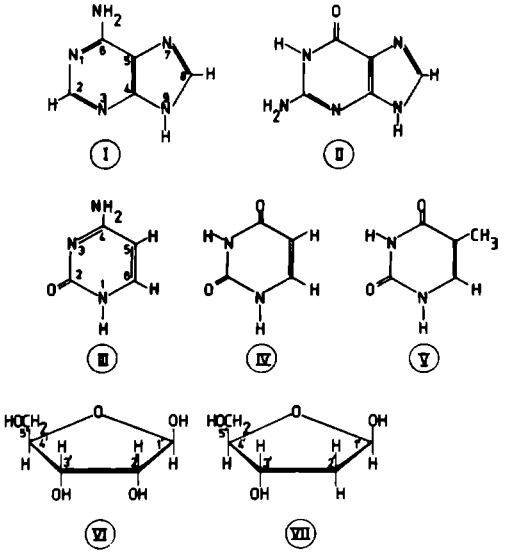
CONTENTS

## INTRODUCTION

Nucleic acids play an essential role in the maintenance of vital processes. Deoxyribonucleic acid (DNA) carries the genetic information, while the various ribonucleic acids (RNA) fulfil important functions in the realization of this information. The nucleic acids are complex molecules built up by extended repetition of nucleotides. Each nucleotide consists of a purine or pyrimidine base, a 5-carbon sugar which is 2'-deoxy-D-ribose for DNA and D-ribose for RNA, and a phosphate group. DNA contains four bases: the purines adenine and guanine and the pyrimidines thymine and cytosine. In RNA thymine is replaced by uracil (Fig. 1). The base is joined to the C-1' position of the sugar by a β-glycosidic linkage, while the phosphate group is esterified at the C-3' and C-5' positions (Fig. 2). According to the model of Watson and Crick (1953), DNA is composed of two anti-parallel polynucleotide chains coiled as a double-helix about a common axis and held together by hydrogen bonds between the bases.

It is generally accepted that DNA is the most critical target for the lethal, mutagenic and carcinogenic effects of ionizing radiation on living organisms. In contrast to U.V. light, which induces damage to DNA mainly by formation of pyrimidine dimers, ionizing radiation damages DNA in many ways including single-strand and double-strand breaks, modification and liberation of the nucleic acid bases, alterations in the sugar moiety, and release of inorganic phosphate (Blok and Loman, 1973; Cerutti, 1976; Hütterman et al., 1978; Latarjet, 1972; Scholes, 1976; Schulte-Frohlinde, 1979; Teoule, 1979; Teoule and Cadet, 1978; Von Sonntag and Schulte-Frohlinde, 1978; Ward, 1975; Ward, 1978).

Although much work has concerned the formation and repair of DNA strand breakage, there is no reason to believe that strand breaks are the only type of biologically significant lesions in DNA. Studies with bacteria, bacteriophages and mammalian cells show that damage to the nucleic acid bases also contributes to the biological effects of ionizing radiation (Cerutti, 1974; Cerutti, 1976). If base damage is not repaired by enzymes it may lead to le-



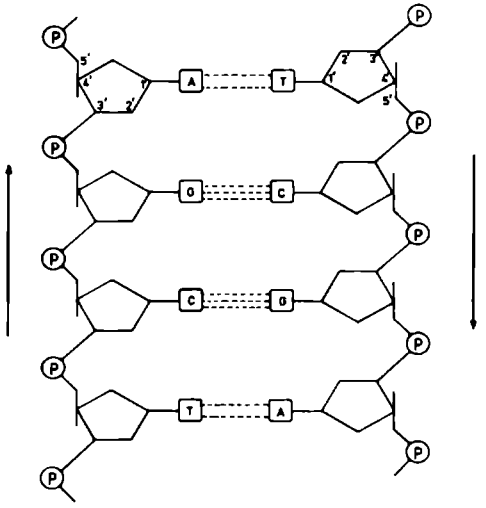


Fig. 1 (left). Structures of DNA and RNA constituents: I adenine, II guanine, III cytosine, IV uracil, V thymine, VI D-ribose, VII 2-deoxy-D-ribose.

Fig. 2 (right). Diagrammatic representation of the polynucleotide chains in DNA.

thality or mutagenesis e.g. when replication or transcription of the altered base occurs or, indirectly, by incorporation of the damaged base into foreign DNA.

While much progress has been made in the chemical characterization of radiation-induced damage to DNA and DNA constituents (Hütterman et al., 1978), it is largely unknown which products are ultimately involved in the impairment by ionizing radiation of the cellular functions leading to cell death, mutation or malignant transformation. In this respect, the radiolytic hydroperoxides of thymine have received much attention. Hahn and Wang (1973) and Cadet and Teoule (1974) identified *cis*-5,6-dihydro-6-hydroperoxy-5-hydroxythymine (6-TOOH) as a major radiation product in irradiated, aerated solutions of thymine. Cadet and Teoule (1975) and Mattern et al. (1973) showed that this product is also formed upon irradiation of thymidine and DNA. Thomas et al. (1976) and Wang et al. (1979) reported the mutagenic action of chemically synthesized 6-TOOH on *Haemophilus influenzae* and *Salmonella typhimurium*. Based on these findings, these authors proposed a molecular mechanism by which ionizing radiation induces mutations *in vivo*.

Although thymine is the most radiation-sensitive base and 6-TOOH has possible mutagenic properties, it cannot be excluded that the large variety of radiolytic products of bases other than thymine, and sugar residues are also involved in the biological effects of ionizing radiation. It would be, however, a tremendous task to isolate and/or synthesize all radiolytic products in quantities large enough to study their individual mutagenicity. With respect to the complexicity of radiochemical events in DNA in aqueous environments as are found in living cells, where numerous chemical changes are to be expected, it appears more worthwhile to study the mutagenicity of gamma-irradiated *solutions* of nucleic acid constituents.

In view of these considerations, the biological consequences of chemical changes in irradiated DNA and DNA constituents might also be of importance for the wholesomeness (safety for human and animal consumption) of irradiated foods. Food irradiation is a rather recent technique developed to preserve foods from microbial and insect damage as well as from physiological deterioration (e.g. sprouting of potatoes, onions; ripening of fruits) thus extending their storage life. Considering the crucial role of nucleic acids in fundamental processes, their high radiosensitivity and the putative mutagenic properties of their radiolytic products, it seems worthwhile to assess the possible deleterious effects of these products in relation to food irradiation.

In the present model study the mutagenicity of gamma-irradiated solutions of nucleic acid bases, nucleosides and sugar moieties has been investigated using various strains of *Salmonella typhimurium* (Ames test) and Chinese hamster ovary cells as indicator organisms. A short description of the effects of ionizing radiation on the nucleic acid constituents and of the short-term test systems used to study mutagenicity will follow now.

## RADIATION CHEMISTRY OF AQUEOUS SOLUTIONS OF NUCLEIC ACID CONSTITUENTS

### *Radiation chemistry of water*

Most biological systems consist of about 80% water. Hence, in dilute aqueous systems most of the radiation energy will be absorbed by the water molecules. As a result of ionization and excitation, water decomposes to the overall equation:

$$H_2O \xrightarrow{\gamma} \quad \cdot OH,\ H\cdot,\ e^-_{aq},\ H_3O^+,\ H_2,\ H_2O_2$$

The radiation chemical yields are expressed by G-values, which refer to the number of species which are produced or disappear per 100 eV of energy absorbed. For practical purposes it is convenient to express the chemical yield as G = 1.04 μmol/l/10 Gy. The yields of the primary products of water radiolysis at neutral pH are given in Table 1. The addition of compounds (scavengers) which react preferentially with one or more of the primary products from water allows control of the type of radicals present in irradiated solutions. For detailed aspects of the radiolysis of water, the reader is referred to Draganic and Draganic (1971), Swallow (1973) and to paper II of this thesis.

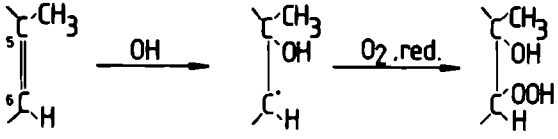
Table 1. G-values for the primary products of water radiolysis at neutral pH.

| $G_{e^-_{aq}}$ | $G_H$ | $G_{OH}$ | $G_{H_2}$ | $G_{H_2O_2}$ |
|---|---|---|---|---|
| 2.65 | 0.55 | 2.70 | 0.45 | 0.70 |

*Radiation chemistry of nucleic acid bases*

In dilute aqueous solutions, radiation damage to the nucleic acid constituents is initiated by attack of the primary radicals (OH, H, $e^-_{aq}$) generated by water radiolysis. The reactivities of these species with the purine and pyrimidine bases are in the order $e^-_{aq}$ > OH > H. Hydrated electrons react with pyrimidines to form radical anions which are rapidly protonated (Scholes, 1963). Typical reactions of OH radicals and H atoms are: abstraction of H atoms and addition to unsaturated centers.

The most widely studied nucleic acid base is thymine. The major radiolytic products in irradiated, aerated solutions of thymine are the hydroperoxides, which are formed by initial addition of an OH radical to the double bond between C-5 and C-6 followed by reaction with molecular oxygen (Teoule and Cadet, 1971; Cadet and Teoule, 1974):



The hydroperoxides decompose to more stable compounds such as 5,6-dihydroxy-5,6-dihydrothymine, 5-hydroxy-5-methylbarbituric acid and urea derivatives.

The formation of hydroperoxides is also observed in irradiated, aerated solutions of uracil and cytosine. They are very unstable and difficult to isolate (Scholes et al., 1960; Latarjet et al., 1963). The main decomposition products of uracil hydroperoxides are 5,6-dihydroxy-5,6-dihydrouracil, isodialuric acid, N'-formyl-$N^2$-formylurea and 5-hydroxyhydantoin (Ducolomb et al., 1973). In irradiated, aerated solutions of cytosine, about 40% of the products are the same as those obtained in the radiolysis of uracil solutions (Polverelli and Teoule, 1974). The main products in irradiated deaerated solutions of the pyrimidines are 5-hydroxy and 5,6-dihydroxy derivatives (Cadet and Teoule, 1971; Shragge et al., 1974; Khattak and Green, 1966).

The knowledge of the radiation chemistry of purines (adenine, guanine) is much less complete than that of the pyrimidines. Guanine has received little attention, because of its low solubility in neutral aqueous solutions. The purines react with OH radicals as rapidly as the pyrimidines. However, their degree of destruction is much lower. Scholes et al. (1960) ascribed this to the occurrence of back reactions of purine-OH radicals reforming the parent purine molecule. In irradiated aerated and deaerated solutions of adenine, the major product is 8-hydroxyadenine, which is formed by attack of the OH radical at the $N_7$-$C_8$ double bond (Conlay, 1963; Van Hemmen and Bleichrodt, 1971). Further degradation of 8-hydroxyadenine leads to allantoxaidine, cyanuric acid, parabanic acid, biuret, triuret and formylurea (Mariaggi and Teoule, 1974). Peroxidation of the 4,5 double bond has been suggested as another mode of adenine degradation, but peroxides have not been identified in irradiated aerated solutions of adenine (Scholes, 1963).

*Radiation chemistry of sugar moieties*

Most of the radiation-chemical changes in DNA and RNA sugars have been characterized by Von Sonntag and co-workers (Von Sonntag and Schulte-Frohlinde, 1978). Upon irradiation of aqueous sugar solutions, primary radicals at all positions in the sugar are formed by abstraction of carbon-bound hydrogen by OH radicals and H-atoms. In oxygenated solutions, these radicals react rapidly with oxygen to give peroxy radicals (see paper II of this thesis). The sugar and sugar-peroxy radicals undergo a series of reactions (disproportionation, rearrangement reactions, elimination of water, C-C bond fragmentation) leading to a variety of end products. In paper II of this thesis a review is given of

the products which have been identified in irradiated oxygenated and deoxygenated solutions of 2-deoxy-D-ribose (Hartmann et al., 1970; Von Sonntag and Schulte-Frohlinde, 1978). Similar and/or identical products to those of 2-deoxy-D-ribose are formed upon irradiation of D-ribose (Von Sonntag and Dizdaroglu, 1977).

*Radiation chemistry of nucleosides and nucleotides*

In irradiated aqueous solutions of nucleosides solvated electrons and H atoms react exclusively with the base moiety. The majority of OH radicals (80%) also react with this part of the nucleosides: the remaining 20% react with the sugar moiety via abstraction of H atoms (Scholes et al., 1960). Thus, radiation-induced damage to nucleosides occurs in the bases as well as in the sugar moiety. Most investigations have been carried out with thymidine. In aqueous deaerated solutions of thymidine four diastereoisomeric forms of 5,6-dihydroxy-5,6-dihydrothymidine are produced (Cadet and Ducolomb, 1974). In aerated solutions of thymidine a total of eight *cis* and *trans* diastereoisomers of 5(6)-hydroperoxy-6(5)-hydroxy-5,6-dihydrothymidine have been detected (Cadet and Teoule, 1972; Cadet and Teoule, 1973), as well as free thymine and thymine derivatives resulting from the rupture of the N-glycosidic bond. Degradation of the unstable hydroperoxides leads to 5,6-dihydroxy derivatives (Cadet and Teoule, 1975). The formation of N-(2-deoxy-β-D-ribofuranosyl) formamide results from rupture of the 1,2 bond in the base moiety.

Dizdaroglu et al. (1976) reported the formation of free altered sugars, as well as release of thymine, in irradiated oxygenated and deoxygenated solutions of thymidine. The structures of these products are identical to those of the radiolytic products found in irradiated solutions of 2-deoxy-D-ribose. Products with an intact base bound to an altered sugar moiety have not been isolated.

The radiation-induced damage to the nucleotides can be divided into four categories. (1) *Breakage of the N-glycosidic bond followed by liberation of the base.* From thymidine-5'-monophosphate (TMP), for example, 2-deoxy-D-ribonic acid 5-phosphate and thymine are the scission products (Cadet et al., 1974). (2) *Damage to the base without rupture of the N-glycosidic bond.* In irradiated oxygenated solutions of TMP and UMP, unstable hydroperoxides are formed. These decompose rapidly to N-formamido nucleotide derivatives (Ducolomb et al., 1974). (3) *Formation of cyclo-nucleotides.* In deaerated solutions of adenosine-5'-monophosphate addition of a C-5' radical to the base moiety leads to the for-

mation of 8,5' cycloadenylic acid (Keck, 1968). (4) *Release of inorganic phosphate*. In deoxygenated solutions, 3'-nucleotides eliminate larger amounts of phosphate than the corresponding 5'-nucleotides (Raleigh et al., 1972; Ward, 1972). Mechanisms for phosphate release have been proposed by Stelter et al. (1976) using D-ribose-5-phosphate as a model compound.

## SHORT-TERM MUTAGENICITY TEST SYSTEMS

There is now increasing evidence that damage to DNA by both physical (e.g. ionizing radiation) and chemical agents in the environment is likely to cause cancer (somatic mutations) and genetic birth defects (heritable germline mutations). Thus, identification of potential carcinogens and mutagens is clearly a major step in reducing human exposure. However, more than 50 000 different chemicals are currently in use and between 500 and 1000 new chemicals are introduced every year. The standard animal tests and human epidemiology alone, are inadequate for detection of carcinogenicity because of time, high costs and the difficulty of dealing with complex mixtures.

In the last two decades numerous short-term testing methods for detecting chemical carcinogens and mutagens have been developed. Recently, Hollstein et al. (1979) gave an excellent review of over 100 assays and discussed the major testing methods in 8 general areas: tests using prokaryotic microorganisms and phages;tests using eukaryotic microorganisms; mutagenicity tests with mammalian cells in culture; tests to measure DNA repair; in vitro transformation tests; in vivo tests in mammals; tests using insects; and mammalian cytogenetic tests in vitro and in vivo. To reduce the number of false positives (non-carcinogens found positive) and false negatives (carcinogens found negative), it is recommended to use a battery of short-term tests.

Two short-term test systems, in particular the Salmonella/mammalian-microsome test (Ames test) and the in vitro Sister-chromatid exchange test with Chinese hamster ovary cells, have been used here to study the mutagenicity of gamma-irradiated solutions of nucleic acid constituents.

### *The Salmonella/mammalian-microsome test*

The most widely used short-term mutagenicity test is the Salmonella/mammalian-microsome test developed by Bruce N. Ames and his colleagues (Ames, 1971; Ames et al., 1973a; Ames et al., 1973b; Ames et al., 1975; Ames, 1979). This test uses several strains of *Salmonella typhimurium* containing different types

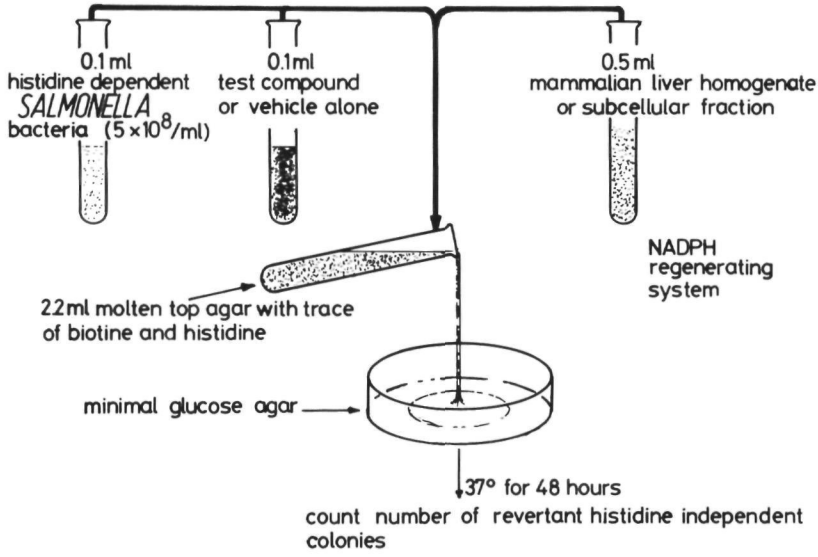



Fig. 3. Performance of the Salmonella/mammalian-microsome test.

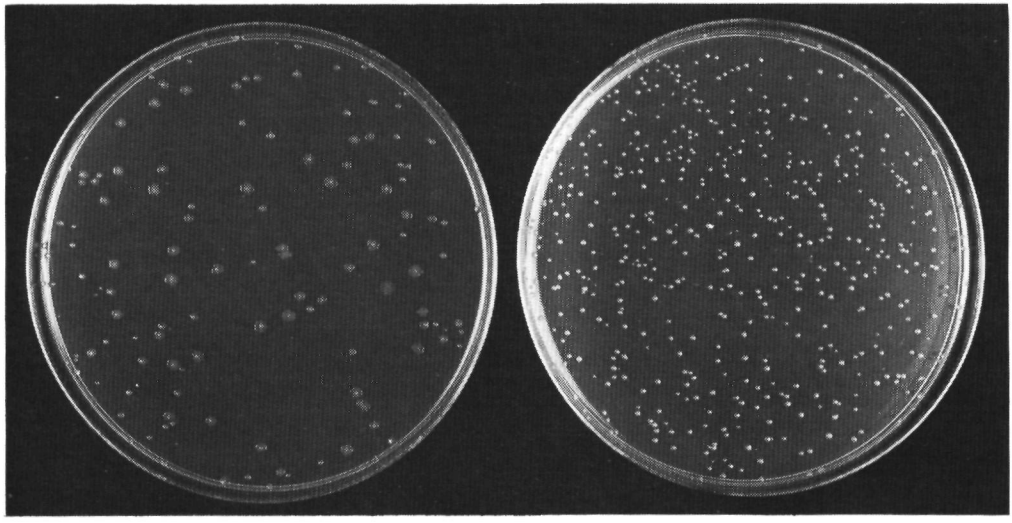


Fig. 4. Photograph showing the reversion of *Salmonella typhimurium* TA 100. Left: spontaneous reversion. Right: reversion induced by 1.0 µg $NaN_3$.

of histidine mutations. This mutation (*his*$^-$) prevents the bacteria from manufacturing one of the enzymes required for the amino acid histidine. As a result the bacteria do not grow unless the medium is supplemented with an excess of histidine.

The *his*$^-$ mutation, however, can be reverted in two ways: spontaneously (the spontaneous rate of reversion is usually very low) or by a chemical which reacts with DNA in such a way that the normal coding sequence of DNA for the required enzyme is restored. The reversion (back mutation) can be scored because *only* revertant bacteria (*his*$^+$) form colonies on a medium that lacks histidine. In other words, this system is suitable to detect chemical mutagens. For general mutagenesis screening Ames et al. (1975) recommend to use five strains of *Salmonella typhimurium*: TA 1535 and TA 100 for the detection of mutagens causing base-pair substitutions; TA 1537, TA 1538 and TA 98 for the detection of various frameshift mutagens.

A number of chemicals must be converted by enzymes in the liver to an active (electrophilic) form before they can react with DNA. By mixing the bacteria with mammalian liver tissue (S9) potential mutagens requiring such metabolic activation can be easily detected (Ames et al., 1973a).

Ames introduced two additional mutations into each strain to enhance its sensitivity to mutagens. One (ΔuvrB) eliminates the capacity for excision repair, so that most of the primary lesions remain unhealed. The other (rfa) causes loss of the lipopolysaccharide barrier (LPS) making the cell more permeable for many chemicals. In addition, the strains TA 100 and TA 98 (derived from TA 1535 and TA 1538 respectively) carry a plasmid (R-factor), a foreign genetic element that makes DNA replication more error-prone. Table 2 shows the genotypes of the five tester strains.

Table 2. Genotypes of the *Salmonella typhimurium* tester strains.

| Histidine mutation | | | Additional mutations | | |
|---|---|---|---|---|---|
| *his* G46 | *his* C3076 | *his* D3052 | LPS | Repair | R-factor |
| TA 1535 | TA 1537 | TA 1538 | rfa | ΔuvrB | - |
| TA 100 | | TA 98 | rfa | ΔuvrB | + |

The practical performance of the Ames test is schematically shown in Fig. 3. The results of such a test (strain TA 100) with sodium azide are presented in Fig. 4.

The simplicity, sensitivity and accuracy of the Ames test has resulted in its world-wide application. It is estimated that about 2.600 chemicals have been so far subjected to this test. In an assay of 300 chemicals, Ames and colleagues (McCann et al., 1975; McCann et al., 1976) found that 90 percent of known chemical carcinogens (from animal experiments) were mutagenic in the Salmonella test, while most non-carcinogens were found to be non-mutagenic. A high correlation between carcinogenicity and mutagenicity has also been reported by several other investigators (Purchase et al., 1978; Sugimura et al., 1976; Sugimura et al., 1977; Brown et al., 1979; Poirier and de Serres, 1979). In an assay of 465 known or suspected carcinogens, divided into 39 categories on basis of their chemical structures, Rinkus and Legator (1979) found an overall correlation of 77% (210/271) for 58% of the compounds (271/465) adequately tested for mutagenicity in Salmonella. For certain classes of chemicals, however, the correlations between carcinogenicity and mutagenicity were rather poor.

*The in vitro Sister-chromatid exchange test*

Sister-chromatid exchanges (SCE s) are the result of a symmetrical exchange between sister chromatids at a homologous locus. The molecular mechanism of exchange is largely unknown (Kato, 1977; Wolff, 1977). SCE s were first described by Taylor (1958) in autoradiographic studies of tritium-labelled plant chromosomes that contained one labelled chromatid and one unlabelled chromatid. More recent procedures involve labelling of the cells (usually Chinese hamster cells or human lymphocytes) with 5-bromodeoxyuridine (BrdUrd), a thymidine analog, for two rounds of replication. After the second mitosis, the metaphase chromosomes consist of one unifilarly-substituted chromatid and one bifilarly-substituted chromatid. These chromatids stain differentially with Giemsa or various fluorescent dyes (Zakharov and Egolina, 1972; Latt, 1973; Korenberg and Freedlender, 1974). The best results are obtained with the fluorescence plus Giemsa (FPG) technique developed by Perry and Wolff (1974).

Induction of SCE s has been proven to be a sensitive indicator of the effects of chemical mutagens and carcinogens on eukaryotic chromosomes (Perry and Evans, 1975). Chemicals that require metabolic activation to become effective mutagens (diethylnitrosamine, dimethylnitrosamine) were found to induce SCE s in CHO cells upon addition of a liver-microsome activating (S9) system (Natarajan et al., 1976; Stetka and Wolff, 1976).

In the present study (see paper IV) a modified BrdUrd-labelling method is applied. Using this technique, sister-chromatid exchanges and chromosome aber-

rations can be scored simultaneously by staining with FPG and Giemsa solutions respectively, in metaphase chromosomes of the first mitosis after the mutagenic treatment.

## SYNOPSIS OF THE THESIS

The results of this study are presented in five papers. Paper I is devoted to the mutagenicity of irradiated, oxygenated, buffered solutions of 2-deoxy-D-ribose, using the Salmonella/mammalian-microsome test. Paper II describes the mutagenicity of irradiated solutions of 2-deoxy-D-ribose and D-ribose as a function of experimental conditions (atmosphere during irradiation, medium, concentration). The results of the mutagenicity studies with irradiated nucleic acid bases and nucleosides are presented in paper III. In paper IV the induction of sister-chromatid exchanges and chromosome aberrations by irradiated, oxygenated solutions of 2-deoxy-D-ribose, thymine and thymidine in CHO cells is described. Finally, paper V describes the formation of adducts between hydrogen peroxide (produced radiolytically or added chemically) and nucleic acid constituents. The radiobiological and mutagenic implications of $H_2O_2$ adduct formation are discussed.

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# MUTAGENICITY OF IRRADIATED SOLUTIONS OF 2-DEOXY-D-RIBOSE

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## INTRODUCTION

Numerous reports exist on the radiation chemistry and biology of nucleic acids and nucleic acid constituents (Ward, 1978; Teoule and Cadet, 1978; Von Sonntag and Schulte-Frohlinde, 1978). However, only little is known of the possible mutagenicity of their radiolytic products. γ-Irradiation, in air with high doses (25 kGy), of thymine and possibly thymidine gives rise to hydroperoxy derivatives (Hahn and Wang, 1973). Recently, it was shown that synthetic preparations of these derivatives are mutagenic to *Salmonella typhimurium* strains TA 100 and TA 98 (Wang et al., 1979). Aiyar and Subba Rao (1977), using a modified Ames test, reported that irradiated solutions of D-ribose (and sucrose) are mutagenic to *S. typhimurium*.

As part of a systematic investigation of the mutagenicity of γ-irradiated solutions of nucleic acid constituents, we report here on the mutagenicity in the Ames test of irradiated, oxygenated, buffered solutions of 2-deoxy-D-ribose. Because, under these conditions, hydrogen peroxide and malonaldehyde are produced in rather high concentrations, we tested them too for mutagenicity, singly and in combination. As discussed later in this paper, these compounds, in the concentrations present in the irradiated 2-deoxy-D-ribose solutions, are not mutagenic.

The 2-deoxy-D-ribose was purchased from Merck. Malonaldehyde was prepared by acid hydrolysis of 1,1,3,3-tetraethoxypropane (Merck). All other chemicals used were of the highest grade available. The *Salmonella typhimurium* tester strains were supplied by Dr. I.E. Mattern (Medical-Biological Laboratory, TNO, Rijswijk, The Netherlands), who received them originally from Dr. B.N. Ames, University of California, Berkeley (U.S.A). The mutability of the strains and their genotypes were regularly checked according to recommended procedures (Ames et al., 1975; De Serres and Shelby, 1979). Sodium azide (TA 100, TA 1535), 9-aminoacridine (TA 1537) and 4-nitro-o-phenylenediamine (TA 98, TA 1538) were used as positive controls. The mutagenicity assay, with and without the liver microsomal fraction (S9), followed that described by Ames et al. (1975).

In some tests, pre-incubation was carried out according to the procedure of Yahagi et al. (1975). The S9 mix contained, per ml: 50 µl S9 (from male Wistar rats induced by Aroclor 1254), 7 µmol $MgSO_4$, 4 µmol glucose 6-phosphate, 4 µmol NADP and 50 µmol phosphate buffer (pH 7.4).

To determine cell survival, appropriate dilutions of the bacterial cultures were made in phosphate-saline buffer and plated along with the test material on minimal plates supplemented with histidine (60 mg per litre medium).

Solutions (0.01 M) of 2-deoxy-D-ribose were prepared in 0.05 M phosphate buffer (pH 7.0) and irradiated at ambient temperature with a $^{60}Co$ γ source (Pilot Plant for Food Irradiation, Wageningen) at a dose rate of 0.12-0.14 kGy/min (1 Gy = 100 rad). Before (30 min) and during irradiation, high-purity oxygen was bubbled through the solutions. Immediately after irradiation, samples were filter-sterilized (HA 0.22 µm, Millipore Corporation, Bedford, MA, U.S.A.) and tested for mutagenicity with strains TA 100, TA 1535 (base-pair mutants), TA 98, TA 1537 and TA 1538 (frameshift mutants). Various volumes of the irradiated and non-irradiated control samples were incorporated in the plates. After incubation of the plates in the dark for 48 h at 37 °C, revertant $his^+$ colonies were counted with a Biotran II Automated Colony Counter.

Hydrogen peroxide was analyzed in the irradiated solutions by the titanium sulphate method of Egerton et al. (1954).

Malonaldehyde assays were made by the thiobarbituric acid procedure of Waravdekar and Saslaw (1959).

*Response of strain TA 100*

As shown in Fig. 1, irradiated, oxygenated, buffered solutions of 2-deoxy-D-ribose were demonstrably mutagenic towards TA 100, giving a clear dose response with radiation as well as with quantities of irradiated sugar. The levels of irradiated 2-deoxy-D-ribose tested did not affect bacterial cell survival. The mutagenicity remained constant during storage at 4 °C under sterile conditions, at least for 3 weeks post-irradiation. Added catalase or S9 had no effect on the mutagenic response, even though 50 µg of hydrogen peroxide per ml was formed immediately post-irradiation (10 kGy) in the oxygenated, buffered solutions of 2-deoxy-D-ribose. The hydrogen peroxide disappeared in the medium with a half-life of about 4 days. Hydrogen peroxide up to 50 µg per plate was not mutagenic to TA 100, but above this level it became toxic to the bacteria. Stich et al. (1978) reported that hydrogen peroxide at non-toxic concentrations, when added for 60 min to a suspension of TA 100 or TA 98, did not significantly increase the frequency of mutations in the Ames test.

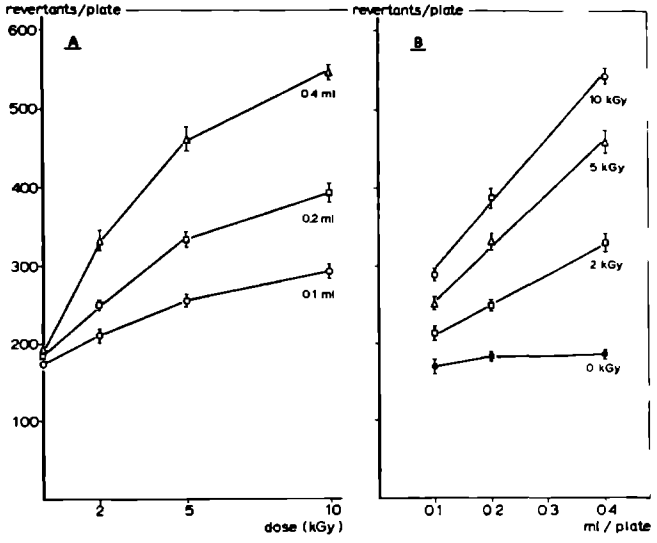


Fig. 1. The mutagenicity of irradiated, oxygenated, buffered solutions of 2-deoxy-D-ribose (0.01 M) to *Salmonella typhimurium* TA 100. Panels A and B demonstrate the mutagenic dose response with both the quantities of 2-deoxy-D-ribose and the dose of radiation. Each point is the average ± S.D. of 3 separate experiments, each involving 3 replicates.

With a radiation dose of 10 kGy we found 20 μg malonaldehyde per ml of the oxygenated solutions of 2-deoxy-D-ribose. Malonaldehyde has been reported (Mukai and Goldstein, 1976; Shamberger et al., 1979) to be mutagenic to several strains of *S. typhimurium*. The highest mutagenicity of malonaldehyde was found with the strains his D 3052 and TA 1978 (Shamberger et al., 1979), both of which are frameshift mutants with a normal excision repair. In our experiments, we noted a doubling of the spontaneous number of revertants of TA 100 at an amount of 3 mg per plate, a level 150 times higher than that produced (per ml) by irradiation with 10 kGy. Equimolar combinations of malonaldehyde and hydrogen peroxide (up to 8 μmol each per plate) were not mutagenic to TA 100. These results, therefore, rule out hydrogen peroxide and malonaldehyde as mutagenic agents in irradiated solutions of 2-deoxy-D-ribose.

*Response of other strains*

Several other strains of the histidine-requiring mutants of *S. typhimurium* were exposed to the irradiated, oxygenated, buffered solutions of 2-deoxy-D-ribose. These included TA 1535, TA 98, TA 1537 and TA 1538. Except for TA 98, no appreciable mutagenicity was observed in the irradiated solutions. Because of the high toxicity of hydrogen peroxide to TA 98 (and other frameshift strains), with reduced survival occurring at levels of 10 μg per plate, the tests were carried out after the addition of catalase or S9, both of which destroy hydrogen peroxide. Against TA 98, the irradiated sugar solution at 0.8 ml/plate with a dose of 10 kGy, gave a slight response, namely a factor of 2.3 over the control level of 17 revertants per plate. Malonaldehyde, tested up to 3 mg per plate, was not mutagenic to strain TA 98.

*Effect of pre-incubation*

When the irradiated (10 kGy), oxygenated, buffered solutions of 2-deoxy-D-ribose were pre-incubated for 20 min, the mutagenic response of TA 100 increased (Fig. 2), attaining a value of nearly double that without pre-incubation at 0.8 ml/plate. In the pre-incubation procedure, however, hydrogen peroxide was toxic to the bacteria at much lower levels than in the normal plate assay. Therefore, pre-incubation experiments were carried out at least one week after irradiation (Fig. 2) when hydrogen peroxide levels were greatly reduced, or after addition of catalase to the irradiated solutions.

A marked increase in mutagenicity was also observed with strain TA 98: the mutagenicity increased from a factor of 2.3 over the controls without pre-in-

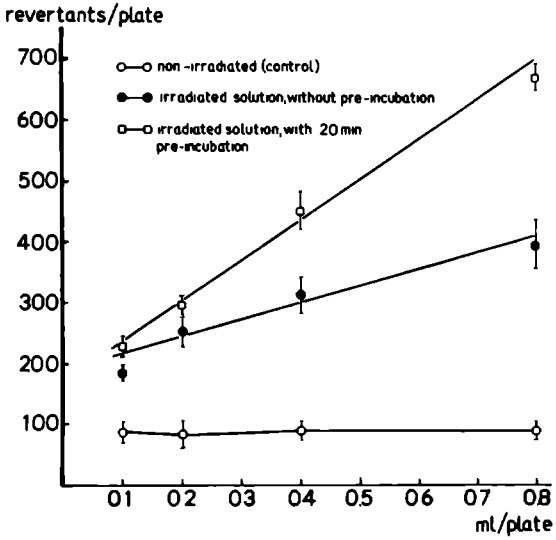


Fig. 2. The mutagenicity of 2-deoxy-D-ribose, irradiated in oxygenated buffered solutions (0.01 M) with a single dose of 10 kGy, to *Salmonella typhimurium* TA 100. Mutagenicity tests were carried out 1 week after irradiation. The control levels (with non-irradiated 2-deoxy-D-ribose) are the same with and without pre-incubation. Each point is the average ± S.D. of 3 replicates.

cubation to a statistically significant higher factor of 4.6 with pre-incubation at 0.8 ml/plate. No induction of mutagenicity was observed with hydrogen peroxide or malonaldehyde upon pre-incubation of strain TA 100 or TA 98.

## DISCUSSION

Most of the reported investigations on the mutagenic or cytotoxic effects of γ-irradiated sugar solutions were performed with non-buffered, aqueous solutions, resulting in a final pH of 3-4 in the irradiated solutions (Schubert, 1969; Kesavan and Swaminathan, 1971; Aiyar and Subba Rao, 1977). Under these conditions, the cytotoxic products of sugar radiolysis consist of α-, β-unsaturated carbonyl sugars derived from radiolytically produced dicarbonyl sugars (hexosuloses) by dehydration or enolization (Schubert and Sanders, 1971; Schubert, 1974). Recently, a series of synthetic uloses have been tested for mutagenicity and one in particular, D-*erythro*-hexose-2,3-diulose, was highly mutagenic to *S. typhimurium* (Van der Linde, private communication, 1979). Bjeldanes and Chew (1979) also reported positive results with 1,2-dicarbonyl compounds in the Ames test.

It is well known that carbonyl and dicarbonyl derivatives (pentosuloses) are formed upon irradiation of oxygen-free, $N_2O$-saturated, aqueous solutions of 2-deoxy-D-ribose (Hartmann et al., 1970). Formation of these compounds is initiated by hydrogen abstraction from the sugar carbon atoms C-3, C-4 and C-5 by the hydroxyl radical, followed by rearrangement reactions and/or elimination of water (Schulte-Frohlinde and Von Sonntag, 1972). In irradiated, oxygenated, aqueous solutions of 2-deoxy-D-ribose, the primary sugar radicals react with oxygen to form peroxy radicals which decay to carbonyl or dicarbonyl derivatives. Some of these are probably the same as found in irradiated, $N_2O$-saturated solutions of 2-deoxy-D-ribose, but up to now, a complete qualitative and quantitative analysis of products of 2-deoxy-D-ribose, irradiated in water in the presence of oxygen, is not available (Von Sonntag and Schulte-Frohlinde, 1978). Neither are data available for irradiated buffered solutions. Our chemical analyses in irradiated, oxygenated, buffered solutions of 2-deoxy-D-ribose show that besides malonaldehyde, about 10 µg of (di)-carbonyl compounds per ml are present compared with about 5 µg/ml in non-buffered solutions. We have found that irradiated non-buffered solutions are mutagenic, without preincubation, but to a lesser extent (about half) than the buffered solutions. In both cases, the mutagenic response is eliminated upon heating or autoclaving the irradiated solutions. This is consistent with the observation that dicarbonyl sugars are easily dehydrated (Theander, 1962).

We hypothesize that the mutagenicity of irradiated, oxygenated solutions of 2-deoxy-D-ribose is due to carbonyl derivatives (uloses or diuloses). Investigations into the isolation and identity of these compounds are currently in progress.

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# MUTAGENICITY OF IRRADIATED OXYGENATED AND DEOXYGENATED SOLUTIONS OF 2-DEOXY-D-RIBOSE AND D-RIBOSE IN *SALMONELLA TYPHIMURIUM*

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SUMMARY

Solutions of 2-deoxy-D-ribose and D-ribose were irradiated under different experimental conditions and tested for mutagenicity, with and without pre-incubation towards *Salmonella typhimurium*. The irradiated sugar solutions gave rise to both base-pair and frameshift mutagenicity. Irradiated, buffered solutions of 2-deoxy-D-ribose were generally more mutagenic than non-buffered solutions. Except for malonaldehyde (MDA), which is non-mutagenic in the concentrations produced radiolytically, the relative mutagenicities of the individual radiolytic products are unknown. However, the observed mutagenicities generally parallel the levels of non-MDA aldehydes.

Heating the irradiated solutions of 2-deoxy-D-ribose resulted in a temperature-dependent reduction of the mutagenicity. Autoclaved, non-irradiated solutions of 2-deoxy-D-ribose were, despite their clastogenicity, non-mutagenic in the Salmonella test.

## INTRODUCTION

The radiation chemistry of DNA and its constituents has been widely studied in order to evaluate the genetic effects of ionizing radiation on a molecular level (Hütterman et al., 1978). The effects of ionizing radiation on nucleic acids in aqueous solutions result from the indirect action of radicals (OH, H and the hydrated electron, $e^-_{aq}$) generated by water radiolysis. Ionizing radiation damages DNA in many ways including single-strand and double-strand breaks, modification and liberation of bases, alterations in the sugar moieties and

release of inorganic phosphate (Blok and Loman, 1973; Cerutti, 1976; Latarjet, 1972; Scholes, 1976; Schulte-Frohlinde, 1979; Teoule, 1979; Teoule and Cadet, 1978; Von Sonntag and Schulte-Frohlinde, 1978; Ward, 1978).

While much progress has been made in the identification and isolation of the radiolytic products from nucleic acid bases, nucleosides, nucleotides and sugar moieties, little information is available on their possible mutagenicity. Thomas et al. (1976) and Wang et al. (1979) demonstrated that synthetic hydroperoxy derivatives of thymine and thymidine were "highly" mutagenic. Since similar compounds are formed upon irradiation of thymine, thymidine and DNA, these authors implicated them in the causation of radiation injury.

Our investigations of the mutagenicity of gamma-irradiated solutions of nucleic acid constituents demonstrated that irradiated (2-10 kGy), oxygenated solutions of 2-deoxy-D-ribose, the sugar moiety of DNA, were mutagenic towards *Salmonella typhimurium* TA 100 and TA 98 (Wilmer et al., 1980). In the present paper we describe the mutagenicity of 2-deoxy-D-ribose and D-ribose, the corresponding sugar in RNA, when irradiated under different experimental conditions which necessarily change the yield and nature of the potentially mutagenic radiolytic products (see below). We also tested autoclaved or heated (both irradiated and non-irradiated) solutions of 2-deoxy-D-ribose since autoclaved solutions of 2-deoxy-D-ribose are highly clastogenic (Schubert, 1974).

*Radiation-chemical considerations*

Within $10^{-9}$s following radiation, the radiolysis of water is represented as follows:

$$H_2O \xrightarrow{\gamma} \cdot OH,\ H\cdot,\ e^-_{aq},\ H_2,\ H_2O_2,\ H_3O^+ \qquad (1)$$

The $e^-_{aq}$ is one of the strongest reducing agents known while the OH radical, produced in equivalent amounts, is one of the most powerful oxidizing agents and is usually responsible for most of the radiation-chemical alterations of organic molecules. It is possible to control the types of radicals present in an irradiated solution by addition of compounds (scavengers) which react preferentially (i.e. high rate constants) with one or more of the radiolytic products from water. In irradiated, oxygenated solutions, the electron and the hydrogen radical are scavenged by molecular oxygen to produce the hydroperoxy (equation 2) and superoxide anion radical (equation 3):

$$H\cdot + O_2 \longrightarrow HO_2\cdot \quad (2)$$

$$e^-_{aq} + O_2 \longrightarrow O_2^-\cdot \quad (3)$$

Unlike the OH radicals, these products react slowly with most organic molecules. In oxygenated solutions, the organic free radicals, produced by OH via H atom abstraction, react with molecular oxygen to form peroxy radicals:

$$RH \xrightarrow{\gamma,\ \cdot OH} R\cdot + H_2O \xrightarrow{O_2} RO_2\cdot \quad (4)$$

The peroxy radicals of 2-deoxy-D-ribose and D-ribose are unstable and decompose rapidly (Von Sonntag and Dizdaroglu, 1977; Von Sonntag and Schulte-Frohlinde, 1978).

In $N_2O$-saturated solutions, the yield of the OH radicals is doubled due to the conversion of $e^-_{aq}$ to OH:

$$e^-_{aq} + N_2O + H_2O \longrightarrow \cdot OH + OH^- + N_2 \quad (5)$$

Unlike $N_2$-saturated solutions in which both $e^-_{aq}$ and OH radicals are present, the $N_2O$-saturated solutions enable investigations to be carried out in an essentially single radical system. If the hydroxyl radical is principally responsible for the production of mutagenic substances, we would expect an approximate doubling of the mutagenic response in irradiated, $N_2O$-saturated solutions of the sugars, compared to irradiations carried out in $N_2$-saturated solutions. In the experiments described here, the mutagenicity tests were performed with 2-deoxy-D-ribose and D-ribose, irradiated in solutions saturated with $N_2$, $N_2O$ or $O_2$.

## MATERIALS AND METHODS

### *Chemicals*

Test compounds were obtained from the following sources: hydrogen peroxide, 2-deoxy-D-ribose, D-ribose and sodium azide from Merck (Darmstadt, West-Germany), 9-aminoacridine and catalase (E.C. 1.11.1.6) from Sigma Chemical Company (St. Louis, MO), 4-nitro-*o*-phenylenediamine and 2-aminoanthracene from Aldrich Europe (Beerse, Belgium). Aroclor 1254 was purchased from NEN Chemicals (Dreieichenhain, West-Germany). Malonaldehyde was prepared by acid

hydrolysis of 1,1,3,3 tetraethoxypropane (Merck). All other chemicals were of the highest purity available.

*Bacterial strains*

*Salmonella typhimurium* strains TA 100, TA 1535 (for the detection of base-pair substitutions), TA 98, TA 1538 and TA 1537 (for the detection of frame-shift mutations) were kindly supplied by Dr. I.E. Mattern (Medical-Biological Laboratory TNO, Rijswijk, The Netherlands). Nutrient broth cultures supplemented with 8% dimethyl sulphoxide were stored under liquid nitrogen. The characteristics of each strain were regularly checked as described by Ames et al. (1975).

*Preparation of S9 fraction*

Male Wistar rats (200-250 g) were injected intraperitoneally with a corn oil solution of Aroclor 1254 (500 mg/kg body weight) 5 days before they were sacrified. Livers were removed aseptically, washed with sterile, ice-cold KCl (0.15 M) and homogenized in 3 vol. of KCl with a Potter-Elvehjem apparatus. The homogenate was centrifuged for 10 min at 9000 g. The supernatant (S9) was collected, divided into 3 ml aliquots and stored in sterile, polypropylene vials at - 196 $^{o}$C. The S9 mix contained, per ml: 7 µmol $MgSO_4$, 4 µmol glucose-6-phosphate, 4 µmol NADP, 50 µmol phosphate buffer (pH 7.4) and 50 µl of the S9 fraction.

*Plate-incorporation assay*

In all experiments, overnight nutrient broth cultures were used. Various volumes (0.1-0.8 ml) of irradiated or non-irradiated (control) test solutions, 0.1 ml of the appropriate bacterial suspension (5 x $10^8$ cells/ml), and in the case of activation, 0.5 ml S9 mix, were mixed in a test-tube containing 2.2 ml of molten top agar (0.6% Bacto agar, 0.5% NaCl, 0.05 M histidine, 0.05 M biotine). The mixture was poured onto minimal agar plates (1.8% Bacto agar, Vogel-Bonner E medium, 2% glucose). After 48 h incubation at 37 $^{o}$C, revertant colonies were counted on a Biotran II Automated Colony Counter. All mutagenicity assays were performed in triplicate. Cell survival was determined by diluting the overnight culture $10^5$ and plating 0.1 ml along with the test solutions and S9 mix on minimal agar plates containing excess histidine (60 mg per litre medium).

*Pre-incubation assay*

The method described by Yahagi et al. (1975) was employed. Cells, test solutions and, when needed, S9 mix were pre-incubated in a shaking incubator (180 rpm) for 20 min at 37 $^{o}$C. After addition of 2.2 ml of molten top agar, the mixture was poured onto minimal agar plates.

*Irradiation*

Solutions of 2-deoxy-D-ribose and D-ribose were prepared with quartz-distilled water (pH 6.0) or with 0.05 M phosphate buffer at pH 7.0. The solutions were irradiated at ambient temperature with a $^{60}$Co gamma-source (Pilot Plant for Food Irradiation, Wageningen, The Netherlands) at a dose rate of 0.12-0.14 kGy/min (1 Gy = 100 rad). A total dose of 10 kGy was given. Before (30 min) and during irradiation, high purity oxygen, nitrogen or nitrous oxide was bubbled through the solutions. As controls, water and phosphate buffer were irradiated under the same conditions. After irradiation, samples were filter sterilized and tested for mutagenicity. Experiments were done at least twice.

*Chemical analyses*

Hydrogen peroxide was analyzed immediately after irradiation by the titanium sulphate method of Egerton et al. (1954). The thiobarbituric acid procedure of Waravdekar and Saslaw (1959) was used for the analysis of malonaldehyde (MDA). The method described by Hartmann (1971) was applied for analysis of non-MDA aldehydes, using thiobarbituric acid as the aldehyde reagent.

RESULTS

*Controls*

The range of spontaneous revertants for each of the five tester strains, as found in our laboratory, is listed in Table 1. The strains were regularly checked for their response to known chemical mutagens: sodium azide for TA 100 and TA 1535, 4-nitro-*o*-phenylenediamine for TA 98 and TA 1538, 9-aminoacridine for TA 1537. To verify the efficiency of the metabolic system, 2-aminoanthracene was used with each strain. Table 1 shows the results, together with control values, obtained in one such experiment.

Table 2. Mutagenicity and cytotoxicity of hydrogen peroxide for *Salmonella typhimurium*.

| Hydrogen peroxide | *His*$^+$ revertants/plate ± S.E.M.[a] | | | | |
|---|---|---|---|---|---|
| (µg/plate) | TA 100 | TA 1535 | TA 98 | TA 1538 | TA 1537 |
| 0.0 | 155 ± 19 (100)[b] | 30 ± 4 (100) | 17 ± 1 (100) | 9 ± 2 (100) | 6 ± 2 (100) |
| 2.5 | - | - | 28 ± 10 (100) | 9 ± 2 (99) | 7 ± 4 (100) |
| 5.0 | 143 ± 29 (100) | 25 ± 3 (100) | 49 ± 10 (79) | 10 ± 3 (99) | 8 ± 2 (95) |
| 7.5 | - | - | 51 ± 9 (45) | 8 ± 1 (76) | 7 ± 2 (95) |
| 10.0 | 149 ± 13 (100) | 25 ± 4 (100) | 47 ± 4 (37) | 6 ± 1 (47) | 15 ± 4 (69) |
| 25.0 | 141 ± 12 (100) | 26 ± 3 (92) | 42 ± 4 (4) | 7 ± 2 (3) | 19 ± 7 (52) |
| 50.0 | 167 ± 14 (73) | 25 ± 1 (75) | 26 ± 8 (1) | 10 ± 1 (1) | 13 ± 3 (19) |
| 75.0 | 181 ± 7 (39) | 28 ± 1 (82) | - | - | - |
| 100.0 | 180 ± 34 ( - ) | 24 ± 3 (64) | - | - | - |

[a]Mean values of two experiments, each performed in triplicate.

[b]Values in parentheses = percentage of surviving cells. Number of revertants was not corrected for survival.

Table 1. Ranges of spontaneous revertants and mutagen specificity for the tester strains of *Salmonella typhimurium*.

| | *His*$^+$ revertants/plate ± S.E.M. | | | | |
|---|---|---|---|---|---|
| | TA 100 | TA 1535 | TA 98 | TA 1538 | TA 1537 |
| Range of controls | 80-180 | 10-30 | 10-35 | 10-20 | 5-15 |
| Control | 95 ± 6 | 15 ± 4 | 26 ± 3 | 10 ± 3 | 10 ± 1 |
| Sodium azide (1.0 µg)[a] | 330 ± 22 | 276 ± 31 | - | - | - |
| 9-aminoacridine (10 µg) | - | - | - | - | 693 ± 42 |
| 4-nitro-o-phenylene-diamine (10 µg) | - | - | 370 ± 24 | 165 ± 11 | - |
| 2-aminoanthracene (1.0 µg) + 25 µl S9/plate | 591 ± 41 | 183 ± 3 | 894 ± 50 | 564 ± 72 | 113 ± 9 |

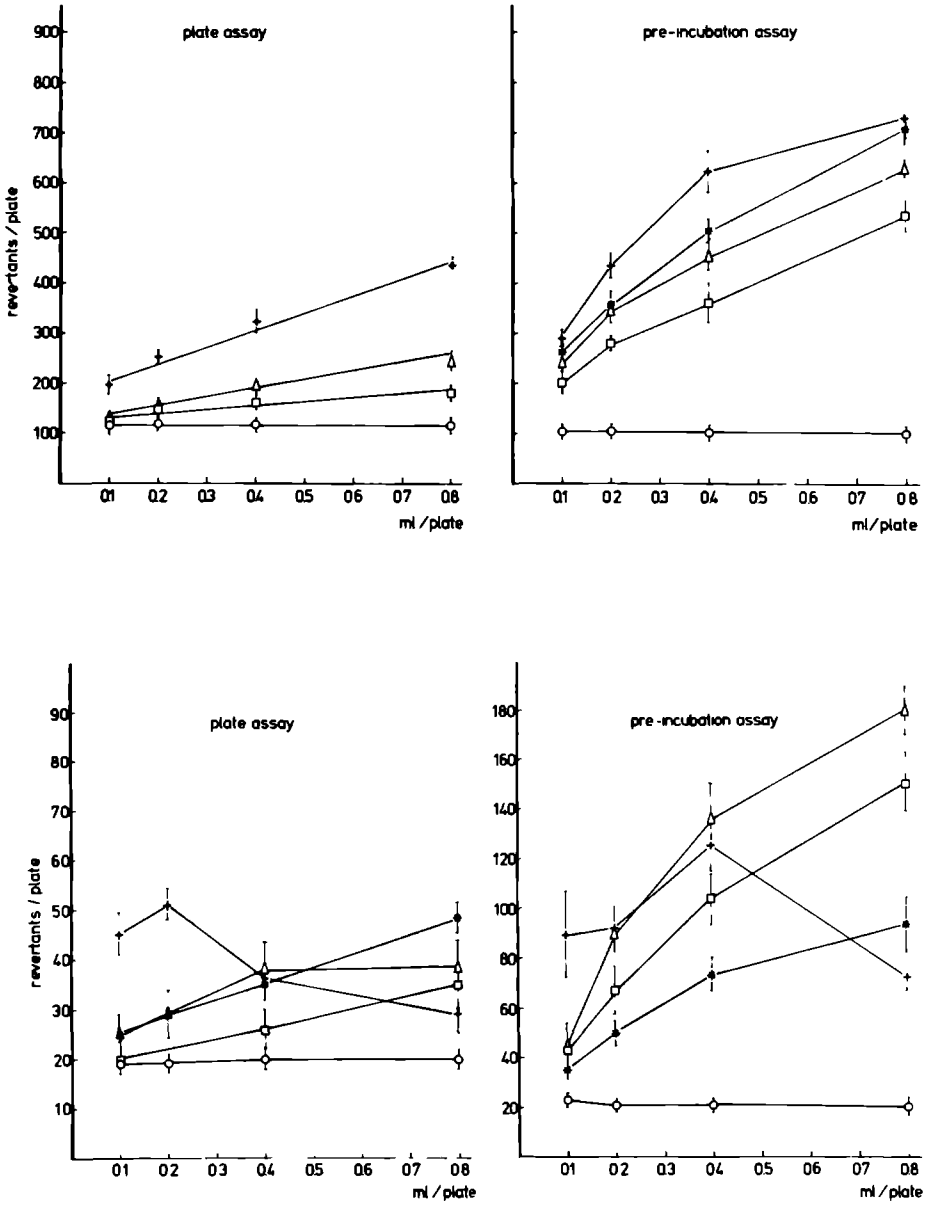
[a]indicates amount per plate

*Hydrogen peroxide*

Hydrogen peroxide is present in irradiated, oxygenated solutions as a primary molecular radiation product. Previously (Wilmer et al., 1980), we summarized briefly the effects of hydrogen peroxide on *Salmonella typhimurium* TA 100 and TA 98. Detailed results of the mutagenicity tests with hydrogen peroxide, using all five tester strains, are presented in Table 2. No mutagenic activity was observed with TA 1535 and TA 1538. A slight increase in the number of revertant colonies was noticed with TA 100 at dose levels which reduced survival. We found a 3-fold increase over the spontaneous mutation rate at a dose of 7.5 µg/plate for TA 98 and at 25 µg/plate for TA 1537. These levels of hydrogen peroxide reduced survival by 50%. Addition of liver homogenate eliminated the mutagenic and cytotoxic effects of hydrogen peroxide. In the pre-incubation assay, cytotoxicity appeared at lower doses of hydrogen peroxide. With the base-pair strains a killing rate of 50% was reached at 25 µg/plate compared to only 5 µg/plate with the frameshift strains.

*Malonaldehyde*

Irradiation of solutions of 2-deoxy-D-ribose produces malonaldehyde (MDA), the amounts of which depend on experimental conditions. MDA was assayed for mutagenicity with strains TA 100 and TA 98. The results, presented in Table 3, show that MDA is not mutagenic for TA 98. Malonaldehyde was found to be a very weak mutagen for TA 100. Addition of S9 mix or pre-incubation had no appreciable effect on the results obtained with both strains. The levels of malonal-




Figs. 1 (above) and 2 (below). Effect of gamma-irradiated (10 kGy), buffered solutions of 2-deoxy-D-ribose (0.01 M) on *Salmonella typhimurium* TA 100 (Fig. 1) and TA 98 (Fig. 2), in plate- and pre-incubation assay. Pooled data (± S.E.M.) of two separate experiments, each performed in triplicate. o–o control (non-irradiated); □–□ $N_2$; Δ–Δ $N_2O$; +–+ $O_2$; *–* $O_2$ + catalase.

Table 3. Mutagenicity and cytotoxicity of malonaldehyde for *Salmonella typhimurium*.

| Malonaldehyde | *His*$^+$ revertants/plate ± S.E.M.[a] | |
|---|---|---|
| (µg/plate) | TA 100 | TA 98 |
| 0.0 | 121 ± 2 (100)[b] | 26 ± 7 (100) |
| 65.0 | 132 ± 3 (100) | 22 ± 11 (100) |
| 325.0 | 147 ± 16 (100) | 27 ± 7 (100) |
| 650.0 | 151 ± 28 (100) | 33 ± 4 (100) |
| 1300.0 | 185 ± 11 (100) | 35 ± 2 (100) |
| 1950.0 | 216 ± 5 (71) | 41 ± 6 (100) |
| 2925.0 | 246 ± 21 (73) | 35 ± 13 (100) |

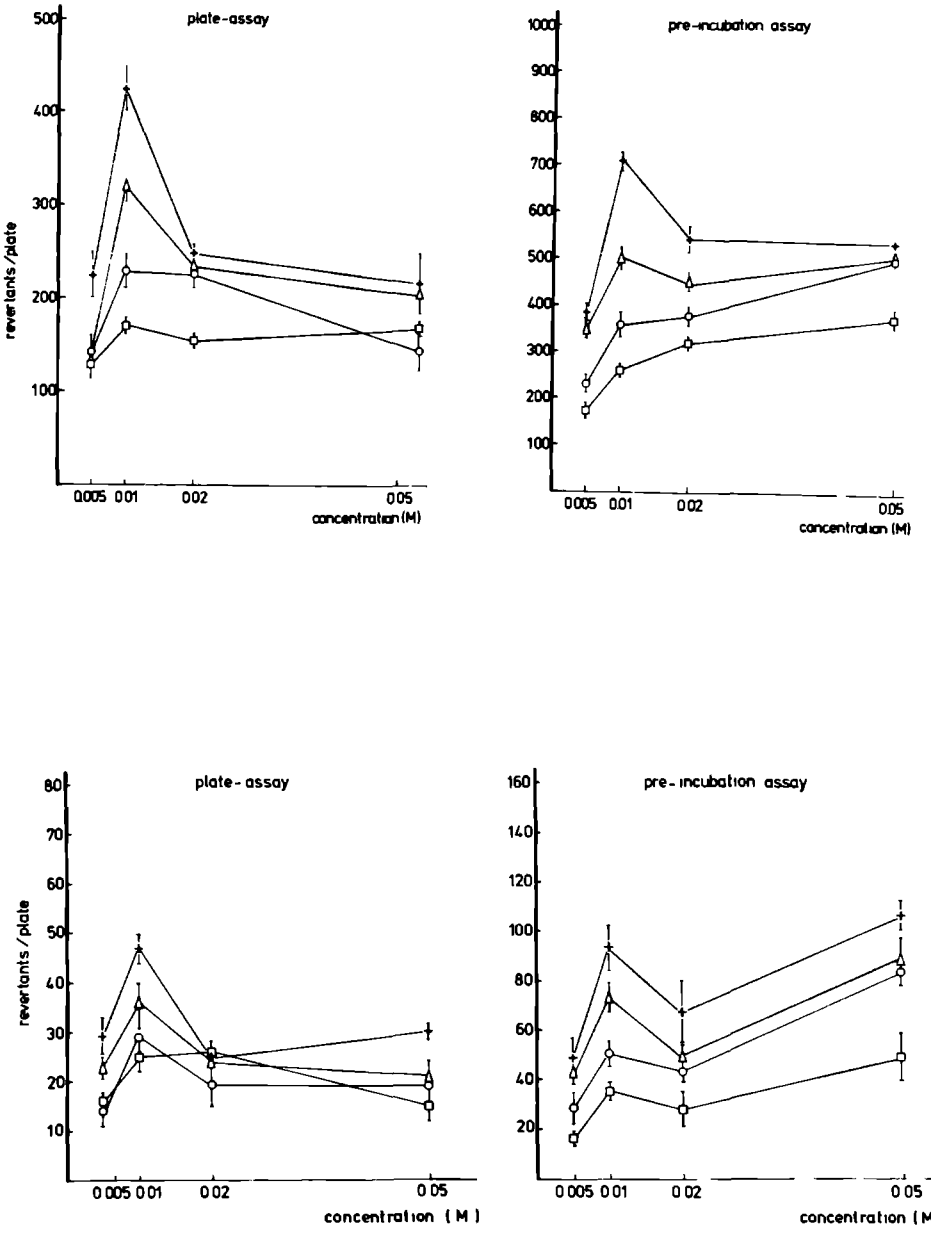
[a] Mean values of two experiments, each performed in triplicate

[b] Values in parentheses = percentage of surviving cells. Number of revertants was not corrected for survival.

dehyde in the irradiated solutions of 2-deoxy-D-ribose (see Table 7) do not exceed 20 µg/ml, which is several orders of magnitude below the highest level listed in Table 3.

*2-deoxy-D-ribose (buffered solutions)*

Fig. 1 shows the dose-response curves for irradiated, buffered solutions of 2-deoxy-D-ribose (0.01 M) with TA 100, in the absence of S9 mix. In the plate-assay a slight increase in the number of $His^+$ revertants was noticed with irradiated, $N_2$-saturated solutions. Irradiated, $N_2O$-saturated solutions doubled the spontaneous mutation rate at 0.8 ml per plate. The irradiated, oxygenated solutions of 2-deoxy-D-ribose exhibit the highest response in both plate- and pre-incubation assay, although in the latter assay the difference with irradiated, $N_2$- and $N_2O$-saturated solutions was far smaller (but still significant) than in the plate-assay. Upon pre-incubation, the irradiated oxygenated solutions, containing approximately 50 µg/ml of hydrogen peroxide, were toxic for Salmonella. At 0.2, 0.4 and 0.8 ml per plate, the survival of the bacteria was reduced to respectively 80, 55 and 30% of the control values. Post-irradiation addition of catalase eliminated these effects. Addition of S9 mix had little or no effect on the mutagenicity of the irradiated solutions of 2-deoxy-D-ribose in the plate-assay. With pre-incubation, however, some de-




Figs. 3 (above) and 4 (below). The mutagenicity of gamma-irradiated (10 kGy) oxygenated buffered solutions of 2-deoxy-D-ribose for *Salmonella typhimurium* TA 100 (Fig. 3) and TA 98 (Fig. 4), in plate- and pre-incubation assay: effect of various sugar concentrations. All solutions were tested for mutagenicity after addition of catalase.
o—o 0.1 ml per plate; □—□ 0.2 ml; Δ—Δ 0.4 ml; +—+ 0.8 ml.

crease (10%) in mutagenicity was observed at 0.8 ml per plate in the presence of S9 mix. As controls, irradiated phosphate buffer (0.05 M; pH 7.0) and non-irradiated solutions of 2-deoxy-D-ribose (0.01 M) were used and found to be non-mutagenic towards TA 100 in both assays.

The results of the mutagenicity assays with TA 98 in the absence of S9 mix are presented in Fig. 2. In the plate-assay irradiated, $N_2$- and $N_2O$-saturated solutions did not show appreciable mutagenicity, while oxygenated solutions gave a weak mutagenic response after post-irradiation addition of catalase. Upon pre-incubation a remarkable increase in mutagenicity was observed, especially with $N_2O$- and $N_2$-saturated solutions (Fig. 2). Note that in both assays the erratic response of oxygenated solutions (not containing catalase) is due to the toxicity of radiolytically produced hydrogen peroxide. Obviously, the reduced survival rates were proportional to the concentration of hydrogen peroxide in the oxygenated solutions.

No mutagenic activity in the irradiated solutions of 2-deoxy-D-ribose was detected using strains TA 1535, TA 1537 or TA 1538.

*Effect of concentration*

The mutagenicity was measured with increasing concentrations of 2-deoxy-D-ribose (0.005, 0.01, 0.02 and 0.05 M) in oxygenated, buffered solutions at a single radiation dose of 10 kGy. Figs. 3 and 4 show the results obtained with TA 100 and TA 98 in plate- and pre-incubation assay. Above 0.005 M the mutagenicity generally increased, though somewhat inconsistently, giving peak values at 0.01 M. However, with pre-incubation and using strain TA 98, peak values occurred at both 0.01 and 0.05 M.

*Effect of heating*

Table 4 summarizes the results of experiments designed to study the effect of heating on the mutagenicity of non-irradiated and irradiated, oxygenated solutions of 2-deoxy-D-ribose (0.01 M in buffer) towards TA 100 (pre-incubation assay). No mutagenicity was induced upon autoclaving the non-irradiated solutions. Upon heating the solutions post-irradiation, a temperature-dependent reduction of the mutagenicity took place. Autoclaving reduced the mutagenicity of the irradiated solutions to practically background values.

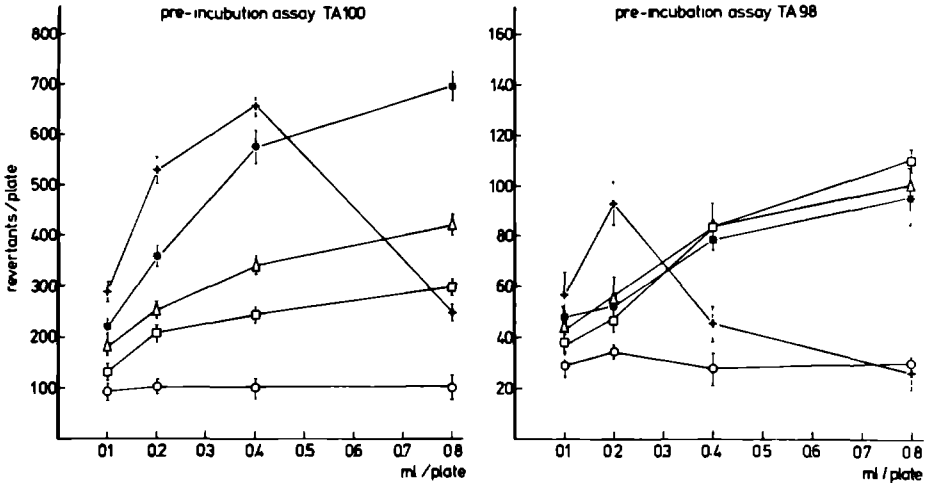



Fig. 5. Effect of gamma-irradiated (10 kGy) non-buffered solutions of 2-deoxy-D-ribose (0.01 M) on *Salmonella typhimurium* TA 100 and TA 98 (pre-incubation assay). Pooled data (± S.E.M.) of two separate experiments.
o—o control (non-irradiated); □—□ $N_2$; Δ—Δ $N_2O$; +—+ $O_2$; ✱—✱ $O_2$ + catalase.

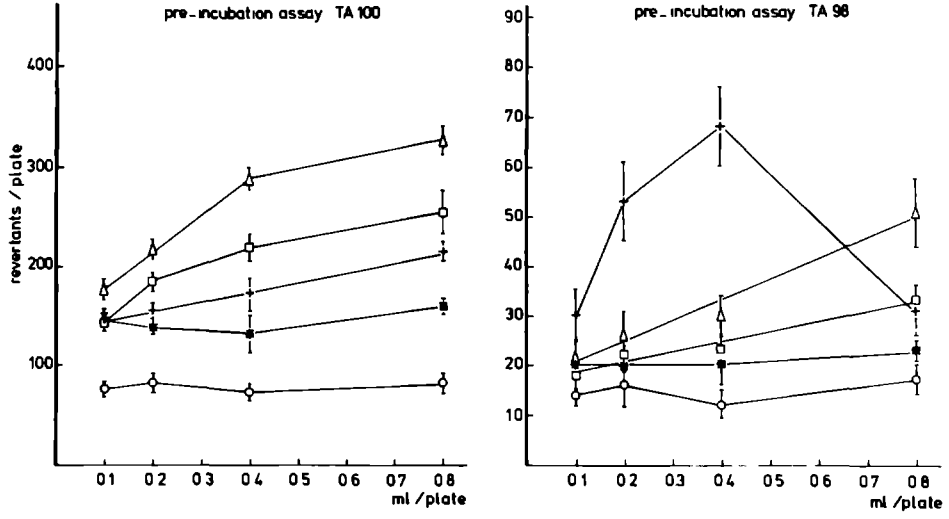



Fig. 6. Effect of gamma-irradiated (10 kGy) buffered solutions of D-ribose (0.01 M) on *Salmonella typhimurium* TA 100 and TA 98 (pre-incubation assay). Pooled data (± S.E.M.) of two separate experiments.
o—o control (non-irradiated); □—□ $N_2$; Δ—Δ $N_2O$; +—+ $O_2$; ✱—✱ $O_2$ + catalase.

Table 4. Mutagenicity of heated, non-irradiated and irradiated, oxygenated solutions of 2-deoxy-D-ribose (0.01 M in buffer) for *Salmonella typhimurium* TA 100 (pre-incubation assay).

| Treatment | | | *His*$^+$ revertants/plate ± S.E.M. | | | |
|---|---|---|---|---|---|---|
| Radiation dose (kGy) | Duration of heating (h) | Temperature (°C) | amount per plate (ml) 0.1 | 0.2 | 0.4 | 0.8 |
| 0 | - | - | 137 ± 12 | 139 ± 10 | 145 ± 7 | 132 ± 14 |
| 0 | 1 | 121[a] | 137 ± 4 | 138 ± 24 | 137 ± 10 | 134 ± 10 |
| 10 | - | - | 334 ± 24 | 523 ± 4 | 702 ± 24 | 912 ± 40 |
| 10 | 1 | 40 | 340 ± 8 | 501 ± 9 | 683 ± 28 | 889 ± 17 |
| 10 | 1 | 70 | 295 ± 30 | 376 ± 24 | 510 ± 5 | 579 ± 6 |
| 10 | 1 | 100 | 139 ± 13 | 156 ± 7 | 191 ± 14 | 218 ± 14 |
| 10 | 1 | 121[a] | 105 ± 11 | 120 ± 11 | 136 ± 11 | 155 ± 11 |

[a]autoclaved (pressure 103 kPa)

*2-deoxy-D-ribose (non-buffered solutions)*

Irradiated (10 kGy), non-buffered solutions of 2-deoxy-D-ribose (0.01 M) were tested for mutagenicity with strains TA 100 and TA 98, using both plate- and pre-incubation assay. Since the pH dropped from 6 to about 4 following irradiation, the pH was adjusted before the mutagenicity assay to 7.0-7.5 with a small volume of 4 M NaOH. With TA 100 (plate-assay) a 2-3 fold increase in the number of spontaneous revertants was noticed at 0.8 ml of irradiated $N_2$-, $N_2O$- and $O_2$-saturated solutions (Table 5). With TA 98, irradiated, oxygenated solutions (containing 68 µg/ml of hydrogen peroxide, which declined in amount down to 45 µg/ml upon raising the pH from 4 to 7) reduced the survival by 80% at 0.8 ml/plate. Addition of catalase to the oxygenated solutions eliminated the cytotoxic effects of hydrogen peroxide. No mutagenic activity towards this strain could be detected neither with irradiated, $O_2$-saturated (plus added catalase) nor with $N_2$- and $N_2O$-saturated solutions (Table 5). In the pre-incubation assay, all three irradiated solutions demonstrated mutagenicity for TA 100 and TA 98 (Fig. 5). With TA 100, the mutagenicity followed the order $O_2 > N_2O > N_2$, while with TA 98, no significant differences in mutagenicity were found between irradiated, $N_2$-, $N_2O$- and $O_2$-saturated solutions. Note again the erratic dose-response curves for oxygenated solutions before addition of catalase.

Table 5. Effect of gamma-irradiated, non-buffered solutions of 2-deoxy-D-ribose (0.01 M) on *Salmonella typhimurium* TA 100 and TA 98 (plate-assay).

| Radiation dose (kGy) | Atmosphere | ml per plate | *His*$^+$ revertants/plate ± S.E.M.[a] TA 100 | TA 98 |
|---|---|---|---|---|
| 0 | - | 0.1 | 83 ± 2 | 32 ± 6 |
| | | 0.2 | 94 ± 8 | 29 ± 3 |
| | | 0.4 | 98 ± 5 | 32 ± 5 |
| | | 0.8 | 96 ± 6 | 30 ± 5 |
| 10 | $N_2$ | 0.1 | 123 ± 6 | 31 ± 2 |
| | | 0.2 | 138 ± 18 | 37 ± 5 |
| | | 0.4 | 179 ± 5 | 35 ± 2 |
| | | 0.8 | 213 ± 8 | 51 ± 2 |
| 10 | $N_2O$ | 0.1 | 139 ± 17 | 30 ± 6 |
| | | 0.2 | 156 ± 11 | 42 ± 3 |
| | | 0.4 | 186 ± 12 | 40 ± 4 |
| | | 0.8 | 242 ± 16 | 56 ± 4 |
| 10 | $O_2$ | 0.1 | 144 ± 10 | 50 ± 4[b] |
| | | 0.2 | 169 ± 13 | 63 ± 2[c] |
| | | 0.4 | 235 ± 15 | 78 ± 12[d] |
| | | 0.8 | 281 ± 10 | 71 ± 6[e] |
| 10 | $O_2$ + catalase | 0.1 | 133 ± 7 | 35 ± 3 |
| | | 0.2 | 170 ± 10 | 42 ± 6 |
| | | 0.4 | 202 ± 14 | 45 ± 6 |
| | | 0.8 | 241 ± 16 | 46 ± 4 |

[a]Mean values of two experiments, each performed in triplicate.
[b,c,d,e]Survival levels reduced to respectively 75, 48, 26 and 10%.

Autoclaved, irradiated or non-irradiated, non-buffered solutions of 2-deoxy-D-ribose were not mutagenic or cytotoxic in the Salmonella assay.

*D-ribose*

Table 6 shows the results (TA 100 and TA 98) obtained with irradiated solutions of D-ribose in the plate-assay. The highest effects were found with $N_2O$- and $N_2$-saturated solutions, which roughly doubled the spontaneous mutation rate of TA 100 at 0.8 ml plate. In the pre-incubation assay (Fig. 6) the mutagenicity of $N_2O$- and $N_2$-saturated solutions was clearly enhanced. Oxygenated solutions which showed a slight, two fold increase in mutagenicity with TA 100 (pre-incubation assay) were found to be non-mutagenic after addition of catalase. With TA 98, severe toxicity was observed in both assays. After addition of catalase, no appreciable mutagenicity could be detected.

Table 6. Effect of gamma-irradiated (10 kGy), buffered solutions of D-ribose (0.01 M) on *Salmonella typhimurium* TA 100 and TA 98 (plate-assay).

| Radiation dose (kGy) | Atmosphere | ml per plate | *His*$^+$ revertants/plate ± S.E.M.[a] TA 100 | TA 98 |
|---|---|---|---|---|
| 0 | - | 0.1 | 84 ± 8 | 13 ± 5 |
| | | 0.2 | 93 ± 15 | 13 ± 3 |
| | | 0.4 | 88 ± 16 | 14 ± 4 |
| | | 0.8 | 78 ± 8 | 12 ± 2 |
| 10 | $N_2$ | 0.1 | 134 ± 16 | 23 ± 7 |
| | | 0.2 | 173 ± 16 | 23 ± 2 |
| | | 0.4 | 154 ± 11 | 16 ± 2 |
| | | 0.8 | 172 ± 8 | 22 ± 7 |
| 10 | $N_2O$ | 0.1 | 114 ± 7 | 14 ± 4 |
| | | 0.2 | 138 ± 9 | 16 ± 6 |
| | | 0.4 | 157 ± 7 | 18 ± 7 |
| | | 0.8 | 196 ± 29 | 28 ± 2 |
| 10 | $O_2$ | 0.1 | 98 ± 3 | 28 ± 2 |
| | | 0.2 | 102 ± 2 | 40 ± 8[b] |
| | | 0.4 | 116 ± 8 | 81 ± 7[c] |
| | | 0.8 | 118 ± 6 | 76 ± 17[d] |
| 10 | $O_2$ + catalase | 0.1 | 105 ± 21 | 12 ± 2 |
| | | 0.2 | 97 ± 5 | 17 ± 6 |
| | | 0.4 | 96 ± 3 | 15 ± 4 |
| | | 0.8 | 118 ± 14 | 30 ± 5 |

[a]Mean values of two experiments each performed in triplicate.

[b,c,d]Survival levels reduced to respectively 81, 63 and 35%. Number of revertants was not corrected for survival.

*Analysis of aldehydes*

Results of the analyses of MDA and non-MDA aldehydes in the irradiated solutions of 2-deoxy-D-ribose are presented in Table 7 (see also Discussion section).

## DISCUSSION

The results presented here clearly show that both types of mutations (base-pair and frameshift) are induced by irradiated solutions of 2-deoxy-D-ribose and D-ribose. This suggests that the mutagenic effects are caused by one or more radiolytic products capable of inducing both base-pair and frameshift mutagenicity. To evaluate the changes in mutagenicity of the irradiated sugar

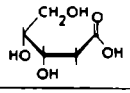
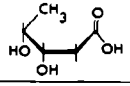
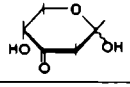
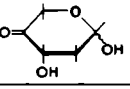
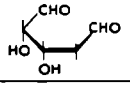
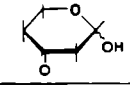
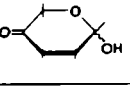
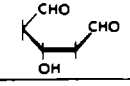
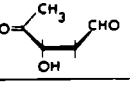
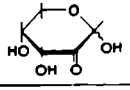
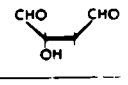
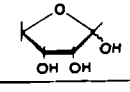
| No | Product | | Aqueous solutions $N_2O$ | Aqueous solutions $N_2O/O_2$ |
|---|---|---|---|---|
| 1 |  | 2-Deoxy-D-ribonic acid (γ- and δ-lactone) | 2.5 | + |
| 2 |  | 2.5-Dideoxy-D-*erythro*-pentonic acid | 0.5 | - |
| 3 |  | 2-Deoxy-D-*glycero*-pentos-3-ulose | 0.2 | + |
| 4 |  | 2-Deoxy-D-*glycero*-pentos-4-ulose | | + |
| 5 |  | 2-Deoxy-D-*erythro*-pentodialdose | | ? |
| 6 |  | 2.4-Dideoxy-pentos-3-ulose | 1.15 | - |
| 7 |  | 2.3-Dideoxy-pentos-4-ulose | 1.0 | - |
| 8 |  | 2.4-Dideoxy-pentodialdose | 0.55 | - |
| 9 |  | 2.5-Dideoxy-D-*glycero*-pentos-4-ulose | | - |
| 10 |  | D-*erythro*-Pentosulose | - | ? |
| 11 |  | 2-Deoxy-D-*glycero*-tetrodialdose | - | + |
| 12 |  | D-Erythrose | - | + |
| 13 | CHO-$CH_2$-CHO | Malonaldehyde | 0.1 | ? |

Fig. 7. Structures and yields of the radiolytic products of 2-deoxy-D-ribose. Yields are expressed as G-values = 1.04 mmol/1/10 kGy. Data taken from Von Sonntag and Schulte-Frohlinde (1978), with permission of the authors and the editor.

Table 7. Levels of malonaldehyde (MDA) and non-MDA aldehydes in irradiated (10 kGy) solutions of 2-deoxy-D-ribose (0.01 M).

| Medium | Atmosphere during irradiation | Total aldehydes (μg/ml) | MDA (μg/ml) | non-MDA aldehydes (μg/ml) |
|---|---|---|---|---|
| buffer | $N_2$ | 22.9 | 15.9 | 7.0 |
| buffer | $N_2O$ | 27.5 | 18.1 | 9.4 |
| buffer | $O_2$ | 32.2 | 20.1 | 12.1 |
| water | $N_2$ | 10.7 | 6.9 | 3.8 |
| water | $N_2O$ | 17.9 | 11.8 | 6.1 |
| water | $O_2$ | 20.9 | 13.6 | 7.3 |

solutions as a function of experimental conditions, it is necessary (1) to have information on the nature and yields of the individual radiolytic products, and (2) to carry out the mutagenicity measurements on each of the radiolytic products. Thanks to the elegant investigations of Von Sonntag and co-workers (Von Sonntag and Dizdaroglu, 1977; Von Sonntag, 1979), it is possible to nearly completely identify the individual radiolytic products formed upon irradiation of non-buffered, $N_2O$-saturated solutions of 2-deoxy-D-ribose and D-ribose and, in part, of $O_2$-saturated solutions.

In irradiated, $N_2O$-saturated solutions of 2-deoxy-D-ribose, the OH radicals attack the various C-atoms producing different sugar radicals leading to the products shown in Fig. 7. We find that none of the sugar acids, produced upon irradiation of 2-deoxy-D-ribose in the crystalline state (Von Sonntag et al., 1974) are mutagenic. In irradiated, oxygenated solutions, the peroxy sugar radicals produce a variety of products though their fate is not fully understood. These radicals probably will undergo bimolecular fragmentation, yielding 4-carbon derivatives. In irradiated, oxygenated solutions of 2-deoxy-D-ribose, the major fragmentation product is 2-deoxy-D-*glycero*-tetrodialdose (Von Sonntag and Schulte-Frohlinde, 1978).

The types of dicarbonyl sugars and compounds having a double bond conjugated with a carbonyl group are known to be highly cytotoxic, some of which are effective anticancer agents (French and Freedlander, 1958; Apple et al., 1970; Schauenstein, 1967). Since malonaldehyde (MDA) itself is not mutagenic at the concentrations produced radiolytically (Table 3), the mutagenicity of the irradiated solutions of 2-deoxy-D-ribose might be reflected in the yield of non-

MDA aldehydes (Table 7). In buffered solutions, the yields of non-MDA aldehydes are higher in $O_2$- and $N_2O$-systems than in $N_2$-systems. The yields in non-buffered solutions are lower in all cases, though again somewhat higher in $O_2$- and $N_2O$-systems than in $N_2$-systems. These results agree with the mutagenicity results in so far as the mutagenicity of the irradiated, buffered solutions is generally higher than that of the non-buffered solutions. Within the buffered or non-buffered systems the mutagenicity of $N_2O$-saturated solutions, at least in the pre-incubation runs, is greater or about equal to that of the $N_2$-saturated solutions, which is in reasonable agreement with the non-MDA aldehyde values (Figs. 1, 2 and 5). Results in $O_2$ are more variable. However, in view of the relatively small differences in mutagenic response; the possibility that the thiobarbituric acid method does not detect all aldehydes; and the fact that the relative mutagenicities of the individual products is unknown, it is not feasible to reach definitive conclusions at this time.

The variations in mutagenicity with the various concentrations of 2-deoxy-D-ribose (Figs. 3 and 4) undoubtedly reflect a variation in the yield and nature of the products formed. With glucose, for example, the radiolytic decomposition increases with increasing sugar concentration, even though the OH radicals are completely scavenged at $5 \times 10^{-3}$ M (Phillips et al., 1966). In irradiated, oxygenated solutions of 2-deoxy-D-ribose, the yield of non-MDA aldehydes increases from 10 µg/ml with 0.005 M to 20 µg/ml with 0.05 M. The nature of the products and their mutagenicity, however can be expected to vary with the sugar concentration since the degree of direct action of ionizing radiation increases with increasing concentration (Phillips et al., 1966).

The decrease in mutagenicity upon heating the irradiated solutions of 2-deoxy-D-ribose supports our hypothesis that the mutagenic effects are due to carbonyl or dicarbonyl compounds. Such compounds are easily dehydrated to e.g. α, β-unsaturated carbonyl derivatives or destroyed upon heating. In our experiments we did not observe any mutagenic or cytotoxic effect in *S. typhimurium* with autoclaved, non-irradiated or irradiated, oxygenated solutions of 2-deoxy-D-ribose. In contrast, the α, β-unsaturated carbonyl compound formed upon heating or autoclaving non-buffered solutions of 2-deoxy-D-ribose has been reported to be highly cytotoxic and clastogenic (Schubert, 1974; Schubert and Sanders, 1971; Esterbauer et al., 1975). Cytotoxicity was observed in buffered suspensions using *S. typhimurium* LT2 as indicator organism and glucose as carbon source. The use of this relatively poor medium might explain these different results.

Our results with irradiated solutions of D-ribose are quantitatively difficult to compare with those of Aiyar and Subba Rao (1977), who reported base-pair as well as frameshift mutagenicity with irradiated, $N_2$-saturated solutions of D-ribose. They used a different procedure, different strains and found low survival, sometimes as little as 0.2%. Therefore their correction factors were so great that it is not possible to make definitive conclusions as to mutagenicity based on these results. The mutagenicity of irradiated solutions of D-ribose is, however, probably due to products similar and/or identical to those found in irradiated solutions of 2-deoxy-D-ribose (Von Sonntag and Dizdaroglu, 1977).

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# MUTAGENICITY OF IRRADIATED SOLUTIONS OF NUCLEIC ACID BASES AND NUCLEOSIDES IN *SALMONELLA TYPHIMURIUM*

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SUMMARY

Solutions of nucleic acid bases, nucleosides and a nucleotide, saturated with either $N_2$, $N_2O$ or $O_2$, were irradiated and tested for mutagenicity towards *Salmonella typhimurium*, with and without pre-incubation. Irradiated solutions of the nucleic acid bases were all non-mutagenic. Irradiated solutions of the nucleosides showed mutagenicity in *S. typhimurium* TA 100 (pre-incubation assay). Generally, the mutagenicity followed the order: $N_2O > N_2 > O_2$. The results demonstrate that the formation of mutagenic radiolytic products is initiated by attack of mainly OH radicals on the 2-deoxy-D-ribose moiety of the nucleosides. With irradiated solutions of the nucleotide, thymidine-5'-monophosphate, no mutagenicity could be detected.

## INTRODUCTION

Ionizing radiation produces many chemical changes in the various constituents of DNA (Teoule and Cadet, 1978; Von Sonntag and Schulte-Frohlinde, 1978). In dilute solutions the damage to DNA and its constituents is initiated by the radicals (OH, H, $e^-_{aq}$) produced by water radiolysis, of which the hydroxyl free radical (OH) is the most important. About 80% of the OH radicals react with DNA by addition to C=C bonds of the nucleic acid bases, while 20% react via attack on the sugar moiety (Scholes et al., 1969).

It is still uncertain which changes in DNA are lethal, mutagenic or carcinogenic. Because of their presumed role in radiation-induced mutagenesis, hydroperoxy products of thymine have received much attention (Cerutti, 1974;

Teoule and Cadet, 1975; Ward, 1975). Recently, Wang et al. (1979) reported that some synthetic hydroperoxy derivatives of thymine and thymidine were mutagenic for *Salmonella typhimurium*. We have demonstrated that gamma-irradiated solutions of 2-deoxy-D-ribose and D-ribose, the sugar moieties of DNA and RNA respectively, are mutagenic towards *S. typhimurium* TA 100 and TA 98 (Wilmer et al., 1980a; Wilmer et al., 1980b).

The present paper deals with the mutagenicities of gamma-irradiated solutions of the nucleic acid bases and their nucleosides. In addition, we also tested the corresponding nucleotide of thymine. This is of special interest in view of the possible role of base damage in radiobiological processes and the fact that the sugar molecule, which is mutagenic upon irradiation, is covalently bound in nucleosides and nucleotides either to the base or to the base and the phosphate group. For reasons given previously (Wilmer et al., 1980b) namely, the relative yields of the primary radicals, irradiation experiments were carried out with $N_2$-, $N_2O$- and $O_2$-saturated solutions of the nucleic acid constituents.

## MATERIALS AND METHODS

### *Chemicals*

All chemicals used were of the highest grade available. Purchases of the following compounds were from the sources indicated: adenine, thymine and uracil (Merck, Darmstadt, West-Germany); cytosine and thymidine (Aldrich Europe, Beerse, Belgium); catalase (E.C. 1.11.1.6), 2'-deoxyadenosine, 2'-deoxycytidine, 2'-deoxyuridine and thymidine-5'-monophosphate (sodium salt) (Sigma Chemical Company, St. Louis, MO, U.S.A.).

### *Bacterial strains*

*Salmonella typhimurium* strains TA 100 and TA 98 were obtained through the courtesy of Dr. I.E. Mattern (Medical-Biological Laboratory, TNO, Rijswijk, The Netherlands).

### *Mutagenesis assay*

Experiments were performed as described previously (Wilmer et al., 1980b), using the plate-incorporation assay of Ames et al. (1975) and the pre-incubation assay of Yahagi et al. (1975). Various volumes (0.1-0.8 ml) of irradiated and non-irradiated (control) solutions were tested. All mutagenicity assays

were done in triplicate. Positive controls ($NaN_3$ for TA 100, 4-nitro-o-phenylenediamine for TA 98) were included in each run. Survival was determined by using $10^{-5}$ dilutions of the bacterial suspension and pouring onto minimal agar plates supplemented with an excess of histidine.

*Irradiation*

Solutions (0.01 M) of nucleic acid bases, nucleosides and nucleotide were prepared with 0.05 M phosphate buffer (pH 7.0). All solutions were irradiated at ambient temperature with a $^{60}Co$ gamma-source (Pilot Plant for Food Irradiation, Wageningen, The Netherlands) at a dose rate of 4 kGy/h. A total dose of 10 kGy was given. Before (30 min) and during irradiation, high purity oxygen, nitrogen or nitrous oxide was bubbled through the solutions. After irradiation, samples were filter sterilized (Millipore, 0.22 μm) and tested for mutagenicity. Experiments were done at least twice.

## RESULTS AND DISCUSSION

Guanine, one of the four bases of DNA, was not considered in this study because of its low solubility at pH 7.0. Irradiated solutions of adenine, cytosine, uracil (RNA base) and thymine were not mutagenic towards TA 100, in plate- and pre-incubation assay as shown in Table 1 (data presented only for 0.8 ml per plate). Negative results were also noted with TA 98. The non-mutagenicity of irradiated, oxygenated solutions of thymine is to be expected in spite of the possible mutagenicity of *cis*-5,6-dihydro-6-hydroperoxy-5-hydroxythymine (6-TOOH). This compound has been identified as a major radiolytic product in irradiated, aerated solutions of thymine (Hahn and Wang, 1973). Wang et al. (1979) reported that five hydroperoxy derivatives of thymine and thymidine (including 6-TOOH) had "extremely high mutagenic activities ranging from 3.2 to 40 revertants per nmole detected with strain TA 100". From their data it would appear unlikely that their compounds were mutagenic in the particular test system employed. For example, they report 9.3 revertants per nmole of 6-TOOH in excess of the control of 165 revertants per plate (no range given). In other words, at the maximum concentration tested (3 nmole of 6-TOOH), they find 28 revertants above the control. However, even if we assume that 6-TOOH is weakly mutagenic as a pure compound, its concentration and stability would be far too low for the irradiated solutions of thymine to exhibit mutagenicity in the Ames test.

Table 1. Effect of gamma-irradiated (10 kGy), buffered solutions of nucleic acid bases (0.01 M) on *Salmonella typhimurium* TA 100.

| Compound (0.8 ml per plate) | Atmosphere during irradiation | *His*$^+$ revertants/plate ± S.E.M. | |
|---|---|---|---|
| | | without pre-incubation | with pre-incubation |
| Control | - | 112 ± 9 | 101 ± 18 |
| Adenine | $N_2$ | 97 ± 19 | 91 ± 14 |
| | $N_2O$ | 111 ± 4 | 103 ± 14 |
| | $O_2$ | 90 ± 4 | 114 ± 8 |
| | $O_2$ (+ catalase)[a] | 94 ± 14 | 121 ± 12 |
| Cytosine | $N_2$ | 108 ± 18 | 120 ± 19 |
| | $N_2O$ | 113 ± 10 | 103 ± 11 |
| | $O_2$ | 129 ± 13 | 130 ± 3 |
| | $O_2$ (+ catalase)[a] | 99 ± 19 | 99 ± 16 |
| Uracil | $N_2$ | 111 ± 7 | 84 ± 12 |
| | $N_2O$ | 112 ± 21 | 118 ± 15 |
| | $O_2$ | 136 ± 8 | 136 ± 11 |
| | $O_2$ (+ catalase)[a] | 105 ± 13 | 94 ± 21 |
| Thymine | $N_2$ | 94 ± 8 | 103 ± 11 |
| | $N_2O$ | 113 ± 6 | 131 ± 3 |
| | $O_2$ | 116 ± 7 | 131 ± 10 |
| | $O_2$ (+ catalase)[a] | 112 ± 6 | 103 ± 4 |

[a]Catalase was added post-irradiation

The results of our mutagenicity assays (TA 100, plate-test) with irradiated solutions of nucleosides are presented in Table 2. Except for irradiated, oxygenated solutions of thymidine, no doubling of the number of spontaneous revertants was found. In the pre-incubation assay (Figs. 1 and 2), irradiated, $N_2O$- and $N_2$-saturated solutions clearly showed mutagenicity. With exception of 2'-deoxyadenosine, $N_2O$-saturated solutions of the nucleosides were more mutagenic than $N_2$-saturated solutions. This indicates that mainly the OH radicals are responsible for the formation of mutagenic radiolytic products. Among the irradiated, oxygenated solutions of nucleosides, which were treated with catalase post-irradiation to destroy hydrogen peroxide, only thymidine and 2'-deoxyuridine (not occurring in DNA but used here as a model compound) demonstrated weak mutagenicity for TA 100. The results of the mutagenicity assays with TA 98 were too inconclusive to draw definite conclusions at this moment.

Irradiated, $N_2O$-, $N_2$- and $O_2$-saturated solutions of thymidine-5'-monophosphate (TMP) were tested with TA 100 and found to be non-mutagenic, both in

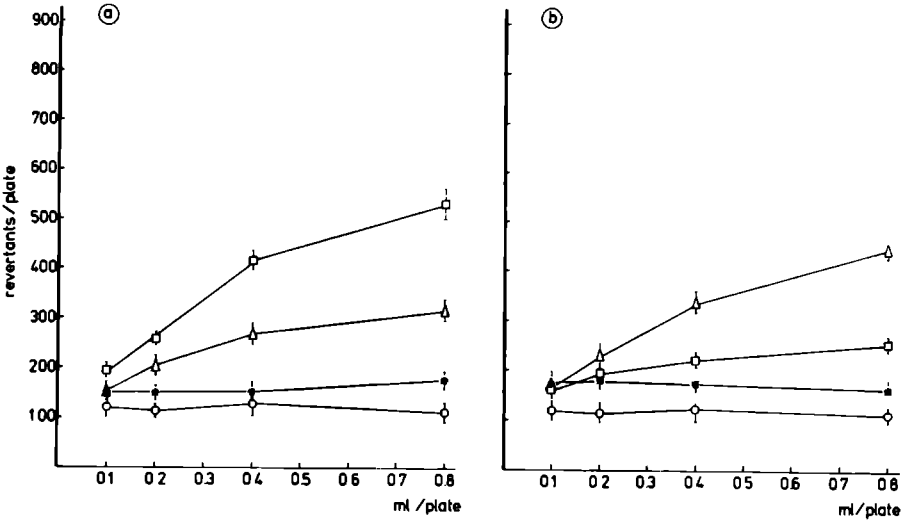


Fig. 1. Mutagenicity of gamma-irradiated (10 kGy) buffered solutions of a) 2'-deoxyadenosine (0.01 M) and b) 2'-deoxycytidine (0.01 M) for *Salmonella typhimurium* TA 100 (pre-incubation assay).
o—o control; □—□ $N_2$; Δ—Δ $N_2O$; ✱—✱ $O_2$ + catalase.

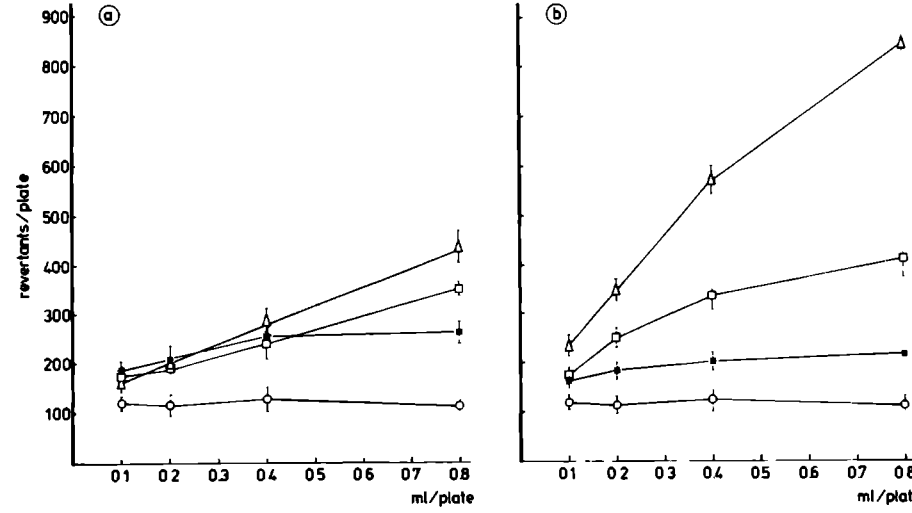


Fig. 2. Mutagenicity of gamma-irradiated (10 kGy) buffered solutions of a) thymidine (0.01 M) and b) 2'-deoxyuridine (0.01 M) for *Salmonella typhimurium* TA 100 (pre-incubation assay).
o—o control; □—□ $N_2$; Δ—Δ $N_2O$; ✱—✱ $O_2$ + catalase.

Table 2. Effect of gamma-irradiated (10 kGy), buffered solutions of nucleosides (0.01 M) on *Salmonella typhimurium* TA 100 (plate assay).

| Compound | Atmosphere during irradiation | *His*$^+$ revertants/plate ± S.E.M. amount per plate (ml) | | | |
|---|---|---|---|---|---|
| | | 0.1 | 0.2 | 0.4 | 0.8 |
| Control | - | 120 ± 19 | 109 ± 9 | 111 ± 10 | 114 ± 4 |
| 2'-deoxyadenosine | $N_2$ | 120 ± 7 | 102 ± 12 | 120 ± 16 | 141 ± 3 |
| | $N_2O$ | 84 ± 2 | 131 ± 14 | 115 ± 5 | 146 ± 7 |
| | $O_2$ | 129 ± 7 | 106 ± 9 | 146 ± 2 | 170 ± 5 |
| | $O_2$ (+ catalase)[a] | 132 ± 11 | 123 ± 10 | 141 ± 1 | 164 ± 4 |
| 2'-deoxycytidine | $N_2$ | 126 ± 10 | 115 ± 13 | 125 ± 16 | 117 ± 12 |
| | $N_2O$ | 132 ± 8 | 122 ± 11 | 130 ± 17 | 124 ± 12 |
| | $O_2$ | 135 ± 12 | 157 ± 16 | 121 ± 11 | 118 ± 16 |
| | $O_2$ (+ catalase)[a] | 114 ± 15 | 123 ± 12 | 127 ± 6 | 135 ± 8 |
| 2'-deoxyuridine | $N_2$ | 116 ± 13 | 102 ± 13 | 133 ± 20 | 145 ± 10 |
| | $N_2O$ | 115 ± 16 | 113 ± 26 | 128 ± 14 | 138 ± 17 |
| | $O_2$ | 135 ± 4 | 156 ± 22 | 197 ± 23 | 215 ± 8 |
| | $O_2$ (+ catalase)[a] | 139 ± 18 | 166 ± 28 | 196 ± 16 | 186 ± 24 |
| thymidine | $N_2$ | 119 ± 6 | 117 ± 2 | 127 ± 14 | 142 ± 17 |
| | $N_2O$ | 132 ± 8 | 125 ± 13 | 133 ± 8 | 144 ± 24 |
| | $O_2$ | 150 ± 14 | 178 ± 26 | 189 ± 24 | 229 ± 12 |
| | $O_2$ (+ catalase)[a] | 141 ± 17 | 157 ± 16 | 187 ± 20 | 250 ± 20 |

[a]Catalase was added post-irradiation

plate- and pre-incubation assay. In Fig. 3 we compared the results obtained with 0.8 ml of the irradiated solutions of TMP with those of thymine and thymidine.

In view of the mutagenicity of irradiated solutions of 2-deoxy-D-ribose (Wilmer et al., 1980a; Wilmer et al., 1980b) and nucleosides, and the non-mutagenicity of irradiated solutions of nucleic acid bases, it may be concluded that the sugar moiety is the main target from which the radicals produce mutagenic radiolytic products. In this respect, Dizdaroglu et al. (1976) showed that upon irradiation of aqueous solutions of thymidine, altered sugars are released by attack of OH radicals on the 2-deoxy-D-ribose moiety resulting in the formation of primary sugar radicals which subsequently undergo reactions leading to transformation of the sugar and to scission of the N-glycosidic bond. These altered sugar products are identical to the products formed upon irradiation of aqueous solutions of 2-deoxy-D-ribose itself (Von Sonntag and Schulte-Frohlinde, 1978). However, aside from the free altered sugars, some contri-

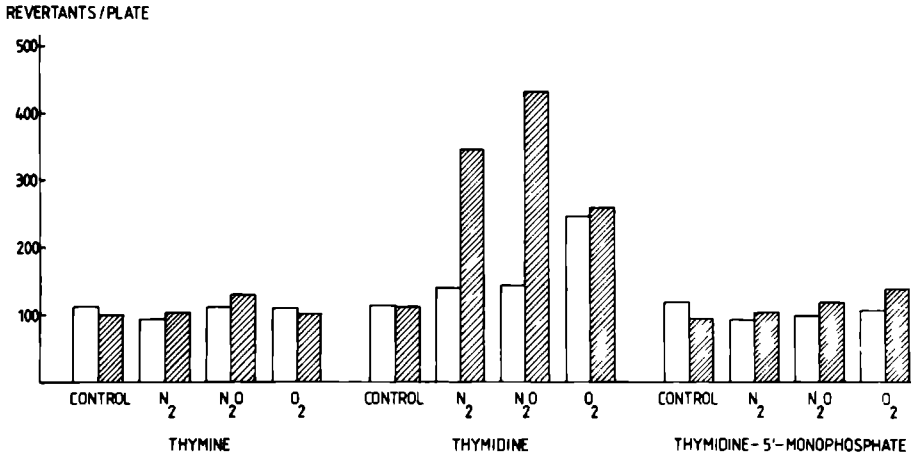


Figure 3. Comparison of the mutagenicities of gamma-irradiated (10 kGy), buffered solutions of thymine, thymidine and thymidine-5'-monophosphate (0.01 M) for *Salmonella typhimurium* TA 100.
□ plate assay; ▨ pre-incubation assay.

butory mutagenic products are probably formed in which the base is not eliminated from the damaged sugar moiety. For example, upon irradiation of aqueous solutions of 2'-deoxyadenosine, modification of the sugar moiety of 2'-deoxyadenosine without liberation of the base has been reported recently (Mariaggi et al., 1979).

Attack of the radicals on the sugar molecule of nucleotides leads to elimination of the phosphate group. This process generally occurs more readily with 3'-nucleotides than with the corresponding 5'-nucleotides (Raleigh et al., 1973; Ward, 1972). However, radiolytic products derived from the deoxyribose moiety have not been found in irradiated solutions of nucleotides. This might be the reason for the non-mutagenicity of the irradiated solutions of TMP.

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# INDUCTION OF SISTER CHROMATID EXCHANGES AND CHROMOSOME ABERRATIONS BY IRRADIATED NUCLEIC ACID CONSTITUENTS IN CHO CELLS

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SUMMARY

Chinese hamster ovary (CHO) cells were exposed to different concentrations of hydrogen peroxide and to gamma-irradiated, oxygenated solutions of thymine, thymidine and 2-deoxy-D-ribose. By using a modified BrdUrd-labelling method sister chromatid exchanges (SCE s) and chromosomal aberrations were scored in the same cell population, one cycle after treatment. Irradiated, oxygenated solutions of 2-deoxy-D-ribose clearly induced SCE s and chromosomal aberrations. In comparison, hydrogen peroxide and irradiated solutions of thymine and thymidine were less effective in CHO cells.

## INTRODUCTION

Radiation-induced changes in the various constituents of DNA (bases, nucleosides, nucleotides, sugars) have been extensively studied in order to elucidate mechanisms leading to cell death or mutation (Teoule and Cadet, 1978; Von Sonntag and Schulte-Frohlinde, 1978). Although many radiolytic products of DNA constituents have been identified now, little is known of their potential adverse effects and their possible role in radiation-induced mutagenesis. Recently, Wang et al. (1979) reported that synthetic hydroperoxy derivatives of thymine and thymidine were mutagenic in *Salmonella typhimurium*. These hydroperoxy compounds have also been identified in irradiated, aerated solutions of thymine and thymidine (Hahn and Wang, 1973).

In a systematic investigation of the mutagenicity of gamma-irradiated solutions of nucleic acid constituents, we showed that irradiated, oxygenated solutions of 2-deoxy-D-ribose, the sugar moiety in DNA, were mutagenic for *Salmonella typhimurium* TA 100 and TA 98 (Wilmer et al., 1980). No mutagenicity by hydrogen peroxide, present as a primary radiolytic product in irradiated, oxygenated solutions, was observed. Irradiated solutions of thymidine were also mutagenic, but to a lesser extent than irradiated solutions of 2-deoxy-D-ribose. No mutagenic effects were found with irradiated solutions of thymine (Wilmer and Schubert, 1980).

The present paper describes the efficiency of hydrogen peroxide and irradiated, oxygenated solutions of thymine, thymidine and 2-deoxy-D-ribose to induce sister chromatid exchanges and chromosomal aberrations in Chinese hamster ovary cells. Induction of sister chromatid exchanges has been shown to be a sensitive indicator of the effects of chemical mutagens and carcinogens on eukaryotic chromosomes (Perry and Evans, 1975). The BrdUrd-labelling method combined with the fluorescence plus Giemsa (FPG) staining technique developed by Perry and Wolff (1974) provides an easy and rapid assay for detecting SCE s and is being routinely used in many laboratories.

In most studies reported cells are grown for two cycles in the presence of BrdUrd to produce TB-BB chromosomes. Thus, after the second mitosis only, the sister chromatids of the metaphase chromosomes will stain differently with the FPG technique. In the experiments described here, the BrdUrd-labelling method was modified according to the way described by Natarajan and Van Kesteren-Van Leeuwen (1980). The mutagenic treatment was given after the labelling of the cells with BrdUrd for one round of replication and followed by a second round of replication in the presence of thymidine to produce TT-TB chromosomes. By this technique, sister chromatid exchanges and chromosome aberrations can be scored simultaneously in metaphase chromosomes of the first mitosis after the mutagenic treatment by staining with FPG and Giemsa solutions respectively.

## MATERIALS AND METHODS

### *Cell line*

Experiments were done with Chinese hamster ovary cells (CHO line) which were grown in Ham's F10 medium containing 15% Newborn calf serum and antibiotics. The modal chromosome number of this cell line is 21-22. The spontaneous frequency of sister chromatid exchanges is 7-8 per cell, when one cycle BrdUrd technique is used for sister chromatid differentiation.

*Chemicals*

Hydrogen peroxide, thymine and 2-deoxy-D-ribose from Merck, and thymidine from Aldrich were used.

*Irradiation*

Solutions (0.01 M) of thymine, thymidine and 2-deoxy-D-ribose were prepared both in water (Milli Q) and in 0.05 M phosphate buffer at pH 7.0. These solutions were irradiated at ambient temperature with a $^{60}Co$ gamma-source (Pilot Plant for Food Irradiation, Wageningen, The Netherlands) at a dose rate of 0.12-0.14 kGy/min (1 Gy = 100 rad). High purity oxygen was bubbled through the solutions during irradiation. A final dose of 10 kGy was given. As controls, water and 0.05 M phosphate buffer were irradiated with the same dose. After irradiation, samples were filter sterilized and stored at 4 $^{o}C$ until use.

*Treatment*

Different concentrations of hydrogen peroxide and irradiated nucleic acid constituents were prepared by diluting them with 10 x F10 medium and water or buffer to give the desired molarity in 1 x F10 medium. When necessary the pH of the solutions was adjusted to 7.2. To exponentially growing cells (in petri dishes) 5-bromo-deoxyuridine (BrdUrd) was given at a final concentration of 10 µM. These cells were allowed to grow in the dark for 12 h at 37 $^{o}C$. At this stage, when cells are grown for one cell cycle in the presence of BrdUrd, the DNA in both chromatids of the metaphase chromosomes has one 5-bromouracil-substituted and one unsubstituted strand (TB-TB). After the prelabelling of the cells with BrdUrd, the medium was removed. The cells were exposed for 2 h to 3 ml of the test solutions (in F10 medium) supplemented with 0.5 ml calf serum. After the treatment, cells were washed with phosphate buffered saline (PBS) (pH 7.2: Ca, Mg free) and incubated for another 16 h in complete F10 medium with thymidine. Two hours before harvesting, colcemid was added to the medium at a concentration of 2 x $10^{-7}$ M. The cells were trypsinized, centrifuged and subjected to a hypotonic shock of sodium citrate (1%) for 10 min. Fixations were done with methanol-acetic acid (3:1) and this fixation step was repeated for at least three times. Air-dried preparations were made on ice-cold wet slides. The chromosomes of the first mitosis after treatment have the TT-TB constitution. With the FPG technique, one chromatid (TT) will stain darkly, its sister chromatid (TB) lightly. Slides were stained then in Hoechst 33258 fluorochrome (15 min, 0.5 µg/ml), immersed in PBS (pH 7.2) and exposed to

white tube light for 16 h. After incubation for 2 h at 65 °C in 5 x SSC (0.75 M sodium chloride - 0.075 M sodium citrate), slides were stained for 10 min in 5% aqueous Giemsa solution (Gurr's R66 improved). SCE s were scored in at least 20 cells. For analysis of chromosomal aberrations slides were stained in Giemsa (6 min, 2%). Chromosomal aberrations including gaps, fragments, breaks, deletions, exchanges, dicentrics and triradials were scored in at least 100 cells.

## RESULTS

The frequencies of SCE s in CHO cells obtained after treatment with hydrogen peroxide, irradiated water and phosphate buffer are presented in Table 1. Statistical analysis of the data with Student's $t$ test did not indicate a significant enhancement ($P > 0.05$) in the frequencies of SCE s, following treatment with irradiated water and phosphate buffer. A slight increase in frequencies of SCE s was observed with hydrogen peroxide up to a concentration of $10^{-3}$ M. Cells were killed after treatment with a concentration of $10^{-1}$ M hydrogen peroxide.

Table 1. Frequencies of SCE s induced by hydrogen peroxide, gamma-irradiated water and phosphate buffer in CHO cells.

| Treatment[a] | Concentration | SCE s/cell[b] | $P$[c] |
|---|---|---|---|
| Control | - | 7.78 ± 0.31 (50)* | |
| Irradiated water | - | 8.50 ± 0.34 (50)* | n.s. |
| Irradiated phosphate buffer | $5 \times 10^{-2}$ M | 7.63 ± 0.27 (49)* | n.s. |
| Hydrogen peroxide | $10^{-4}$ M | 8.33 ± 0.31 (46)* | n.s. |
| | $5 \times 10^{-4}$ M | 9.36 ± 0.49 (25) | s. |
| | $10^{-3}$ M | 10.18 ± 0.57 (45)* | s. |
| | $10^{-2}$ M | 9.00 ± 0.68 (22) | s. |
| | $10^{-1}$ M | no surviving cells | |
| 4-nitroquinoline-N-oxide | 2.27 µg/ml | 53.2 ± 4.0[d] | |

[a] Treatment for 2 h
[b] Means ± S.E.M. In parentheses: number of cells scored
[c] Significant (s.) at 0.05 level
[d] Data from Natarajan and Van Kesteren-van Leeuwen (1980)
* Results from 2 experiments

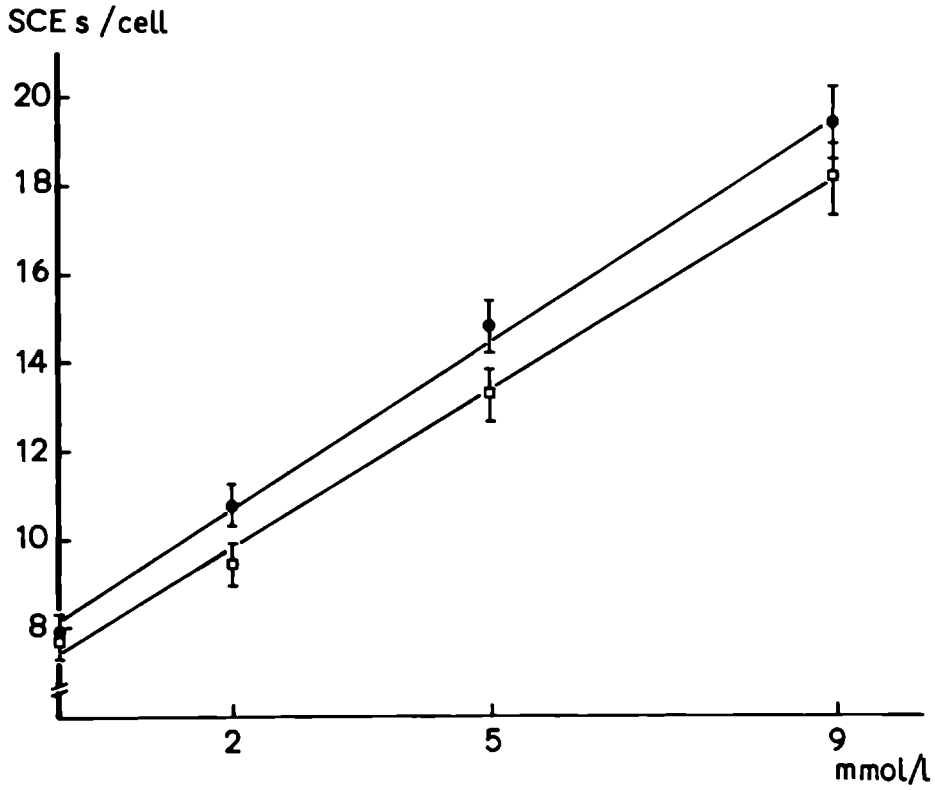


Figure 1. Sister chromatid exchanges induced by gamma-irradiated (10 kGy), oxygenated solutions of 2-deoxy-D-ribose (0.01 M). Ordinate gives the concentration of 2-deoxy-D-ribose.
●—● irradiated in water; □—□ irradiated in buffer.

Figs. 1 and 2a show the frequencies of SCE s induced by irradiated solutions of 2-deoxy-D-ribose. The frequencies of SCE s increased linearly with the concentration of 2-deoxy-D-ribose. Non-buffered solutions were more effective in inducing SCE s than buffered solutions (Fig. 1). Data pertaining to irradiated solutions of thymine and thymidine are presented in Table 2. Irradiated solutions of thymine (both in water and in phosphate buffer) induced a significant increase ($P < 0.005$) in frequencies of SCE s at concentrations of respectively 5 and 9 x $10^{-3}$ M. The frequencies of SCE s in cells treated with irradiated solutions of thymidine were significantly higher than the control at all concentrations used ($P < 0.005$), but did not increase linearly with the concentration of thymidine.

Table 2. Frequencies of SCE s induced by gamma-irradiated solutions of thymine and thymidine in CHO cells.

| Treatment[a] | Concentration | SCE s/cell[b] | $P^c$ |
|---|---|---|---|
| Control | - | 7.16 ± 0.27 (25) | |
| Non-irradiated thymine (water) | $9 \times 10^{-3}$ M | 7.48 ± 0.36 (25) | |
| Irradiated thymine (water) | $2 \times 10^{-3}$ M | 7.64 ± 0.48 (25) | n.s. |
| | $5 \times 10^{-3}$ M | 11.00 ± 0.56 (36) | s. |
| | $9 \times 10^{-3}$ M | 12.33 ± 0.96 (25) | s. |
| Non-irradiated thymine (buffer) | $9 \times 10^{-3}$ M | 7.44 ± 0.40 (25) | |
| Irradiated thymine (buffer) | $2 \times 10^{-3}$ M | 8.29 ± 0.52 (25) | n.s. |
| | $5 \times 10^{-3}$ M | 9.31 ± 0.30 (20) | s. |
| | $9 \times 10^{-3}$ M | 10.52 ± 0.58 (31) | s. |
| Non-irradiated thymidine (water) | $9 \times 10^{-3}$ M | 7.30 ± 0.37 (25) | |
| Irradiated thymidine (water) | $2 \times 10^{-3}$ M | 10.43 ± 0.51 (30) | s. |
| | $5 \times 10^{-3}$ M | 12.86 ± 0.76 (30) | s. |
| | $9 \times 10^{-3}$ M | 12.07 ± 0.67 (25) | s. |
| Non-irradiated thymidine (buffer) | $9 \times 10^{-3}$ M | 7.36 ± 0.37 (25) | |
| Irradiated thymidine (buffer) | $2 \times 10^{-3}$ M | 10.36 ± 0.83 (22) | s. |
| | $5 \times 10^{-3}$ M | 11.70 ± 0.79 (25) | s. |
| | $9 \times 10^{-3}$ M | 11.71 ± 0.54 (30) | s. |

[a] Treatment for 2 h
[b] Means ± S.E.M. In parentheses: number of cells scored
[c] Significance (s.) at 0.005 level

Tables 3 and 4 summarize the results of the chromosome aberration analysis. No induction of chromosomal aberrations was detected with irradiated water and phosphate buffer, while the frequency of aberrations induced by hydrogen peroxide was significantly higher only at a concentration of $10^{-2}$ M. As shown in Table 3 and Fig. 2b, irradiated, non-buffered solutions of 2-deoxy-D-ribose clearly enhanced the frequencies of both breaks (chromatid and isochromatid breaks, chromatid and isochromatid gaps, fragments and interstitial deletions) and exchanges (chromatid exchanges, dicentrics and triradials). Both types of aberrations were concentration dependent. The frequencies of breaks showed a linear relationship with the concentration. The frequencies of exchanges increased more than linearly with the concentration of 2-deoxy-D-ribose. In comparison to non-buffered solutions, irradiated buffered solutions of 2-deoxy-D-ribose were less efficient in inducing chromosomal aberrations.

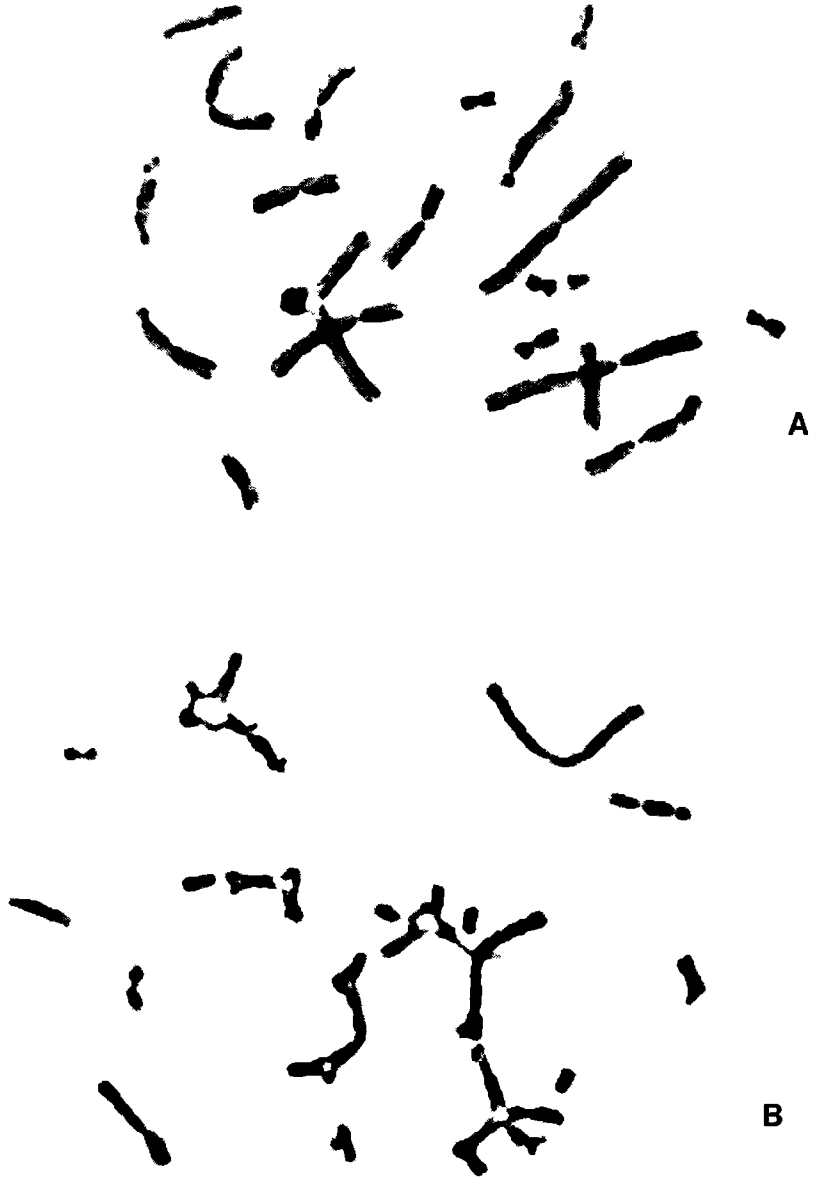


Fig. 2. Sister-chromatid exchanges (a) and chromosomal aberrations (b) induced by gamma-irradiated (10 kGy) oxygenated solutions of 2-deoxy-D-ribose (0.01 M) in CHO cells.

Table 3. Frequencies of chromosome aberrations induced by hydrogen peroxide, gamma-irradiated water, phosphate buffer and solutions of 2-deoxy-D-ribose in CHO cells.

| Treatment | Concentration | Number of cells scored | Aberrations/100 cells | |
|---|---|---|---|---|
| | | | Breaks[a] | Exchanges[b] |
| Control | - | 100 | 11 | 0 |
| Irradiated water | - | 100 | 13 | 0 |
| Irradiated phosphate buffer | $5 \times 10^{-2}$ M | 100 | 7 | 0 |
| Hydrogen peroxide | $10^{-4}$ M | 100 | 8 | 0 |
| | $5 \times 10^{-4}$ M | 100 | 7 | 1 |
| | $10^{-3}$ M | 100 | 6 | 1 |
| | $10^{-2}$ M | 100 | 13 | 15 |
| | $10^{-1}$ M | no surv. cells | | |
| Non-irradiated 2-deoxy-D-ribose (water) | $9 \times 10^{-3}$ M | 144 | 4.2 | 0.0 |
| Irradiated 2-deoxy-D-ribose (water) | $2 \times 10^{-3}$ M | 200 | 7.0 | 0.5 |
| | $5 \times 10^{-3}$ M | 200 | 17.0 | 11.5 |
| | $9 \times 10^{-3}$ M | 191 | 25.7 | 65.4 |
| Non-irradiated 2-deoxy-D-ribose (buffer) | $9 \times 10^{-3}$ M | 100 | 11 | 0 |
| Irradiated 2-deoxy-D-ribose (buffer) | $2 \times 10^{-3}$ M | 100 | 9 | 0 |
| | $5 \times 10^{-3}$ M | 100 | 11 | 0 |
| | $9 \times 10^{-3}$ M | 100 | 24 | 8 |

[a]Breaks include both chromatid and isochromatid breaks
[b]Exchanges include all types of interchanges including triradials

Aberrations induced by irradiated solutions of thymine and thymidine did not indicate a clear-cut relationship to the concentrations used (Table 4). The same phenomenon as described above was observed with non-buffered and buffered solutions. Irradiated, non-buffered solutions of both thymine and thymidine showed a higher response than the corresponding buffered solutions.

## DISCUSSION

The results presented here clearly show that the one cycle BrdUrd-labelling method can be successfully used (Fig. 2). In our experiments hydrogen peroxide itself enhanced the frequencies of SCE s only slightly. MacRae an Stich (1979) have tested hydrogen peroxide at low concentrations ($10^{-7}$ and $10^{-4}$ M) in CHO cells and found it to increase the frequencies of SCE s to a greater extent

Table 4. Frequencies of chromosomal aberrations induced by gamma-irradiated solutions of thymine and thymidine in CHO cells.

| Treatment | Concentration | Number of cells scored | Aberrations/100 cells | |
|---|---|---|---|---|
| | | | Breaks[a] | Exchanges[b] |
| Non-irradiated thymine (water) | $9 \times 10^{-3}$ M | 150 | 5.3 | 0.7 |
| Irradiated thymine (water) | $2 \times 10^{-3}$ M | 100 | 9 | 0 |
| | $5 \times 10^{-3}$ M | 100 | 17 | 12 |
| | $9 \times 10^{-3}$ M | 26 | 15.4 | 3.8 |
| Non-irradiated thymine (buffer) | $9 \times 10^{-3}$ M | 100 | 13 | 1 |
| Irradiated thymine (buffer) | $2 \times 10^{-3}$ M | 100 | 6 | 3 |
| | $5 \times 10^{-3}$ M | 100 | 9 | 0 |
| | $9 \times 10^{-3}$ M | 100 | 27 | 10 |
| Non-irradiated thymidine (water) | $9 \times 10^{-3}$ M | 150 | 9.3 | 0.0 |
| Irradiated thymidine (water) | $2 \times 10^{-3}$ M | 100 | 5 | 1 |
| | $5 \times 10^{-3}$ M | 100 | 21 | 14 |
| | $9 \times 10^{-3}$ M | 50 | 12 | 12 |
| Non-irradiated thymidine (buffer) | $9 \times 10^{-3}$ M | 100 | 7 | 0 |
| Irradiated thymidine (buffer) | $2 \times 10^{-3}$ M | 100 | 14 | 0 |
| | $5 \times 10^{-3}$ M | 100 | 5 | 1 |
| | $9 \times 10^{-3}$ M | 100 | 7 | 1 |

[a]Breaks include both chromatid and isochromatid breaks
[b]Exchanges include all types of interchanges including triradials

than we did. However, it is very difficult to compare their results with ours, because they treated the cells for respectively 24 and 3 h, using the two cycles BrdUrd-labelling method. The capability of hydrogen peroxide to induce chromosomal aberrations has been reported by several authors. Schöneich (1967) reported induction of chromosomal aberrations by hydrogen peroxide in strains of ascites tumors in mice. Stich et al. (1979) found a relatively high frequency of chromosomal aberrations in CHO cells (without BrdUrd-labelling) and a marked DNA repair synthesis in cultured human fibroblasts.

The hydrogen peroxide concentrations in irradiated, oxygenated, non-buffered and buffered solutions of 2-deoxy-D-ribose (0.01 M) were respectively 2.1 and $1.5 \times 10^{-3}$ M immediately post-irradiation. Upon storage at 4 $^{o}$C the hydrogen peroxide concentration in buffered solutions decreased with a half life of

about 4 days (Wilmer et al., 1980). By the time we did the experiments (2 weeks after irradiation), the peroxide content was greatly reduced. The different capability of irradiated non-buffered and non-buffered solutions of 2-deoxy-D-ribose to induce SCE s and, in particular, chromosomal aberrations cannot be due to a difference in the concentration of hydrogen peroxide, because:
(a) by raising the pH (4.0) of irradiated, non-buffered solutions to 7.2, the hydrogen peroxide concentration declined from 2.1 to about $1.2 \times 10^{-3}$ M,
(b) at that concentration as well as the actual concentration in the buffered solutions, hydrogen peroxide itself is hardly effective in our system.

The present results are so far comparable with those obtained with the Salmonella test, that irradiated solutions of 2-deoxy-D-ribose are positive in both test systems (Wilmer et al., 1980). However, using the Ames' plate-incorporation assay with *Salmonella typhimurium* TA 100 we found a lower mutagenic response with non-buffered solutions than with buffered solutions of 2-deoxy-D-ribose. This difference greatly disappeared when the solutions were pre-incubated. The reasons for the higher frequencies of SCE s and, especially, chromosomal aberrations induced by irradiated, non-buffered solutions of 2-deoxy-D-ribose in CHO cells are not clear. As a wide variety of products is formed upon irradiation of 2-deoxy-D-ribose (Von Sonntag and Schulte-Frohlinde, 1978), possibly a number of radiolytic products has mutagenic activity with differential response towards bacterial and mammalian cells.

Irradiated solutions of thymidine are less efficient than irradiated solutions of 2-deoxy-D-ribose in inducing SCE s and chromosomal aberrations. A similar trend to that was observed in the Salmonella test. Irradiated solutions of thymine, however, are weakly positive in CHO cells, while negative results were obtained in the Salmonella assay (Wilmer and Schubert, 1980).

It should be clarified, whether the enhancement of the frequencies of SCE s as found here e.g. with irradiated solutions of thymine and thymidine must be considered as a significant effect or not. Up to now, the criteria to define a compound positive by the SCE test vary from laboratory to laboratory. It is important to generate standard protocols for this test system and define criteria and appropriate statistical treatment as has been done for the Salmonella test (De Serres and Shelby, 1979). This would be helpful in the interpretation of data and comparison of results of the various laboratories using the SCE technique.

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# HYDROGEN PEROXIDE ADDUCTS OF NUCLEIC ACID CONSTITUENTS

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SUMMARY

It is shown that low concentrations ($\sim 10^{-4}$ M) of $H_2O_2$, whether produced radiolytically or added chemically, form stable, chelate-like hydrogen-bonded adducts with amino acids, nucleic acid bases and nucleosides and other molecules capable of polyfunctional hydrogen bonding. These $H_2O_2$ adducts are detected and their relative stabilities determined by a sugar competition procedure in which the rate of decomposition of $H_2O_2$ is measured in the presence and absence of test molecules. In glucose media the order of stability of the peroxide adducts is: adenine > thymine > cytosine > uracil, while in fructose media the position of thymine and cytosine is reversed. With nucleosides the sugar moiety governs the rates of breakdown of $H_2O_2$. Several chemical and physical factors which could influence the stabilities of the $H_2O_2$ adducts were investigated including different concentrations of nucleic acid constituents and added metal ions, free and complexed. The radiobiological and mutagenic implications of $H_2O_2$ adduct formation are explored.

## INTRODUCTION

Studies on the chemical and biological properties of irradiated aqueous media suggested that in the presence of low concentrations of hydrogen peroxide ($\sim 10^{-4}$ M), produced radiolytically or added chemically, molecules capable of polyfunctional hydrogen bonding form stable, chelate-like 1:1 hydrogen bonded adducts (Dirscherl and Mosebach, 1954; Schubert et al., 1969; Schubert, 1974). These $H_2O_2$ adducts, some of which have been prepared in pure form (Dirscherl and Mosebach, 1954; Schubert, 1974) possess cytotoxic activity, e.g. on the

growth of bacteria (Schubert et al., 1969) and ascites tumor cells (Weitzel et al., 1961), and on isolated rat thymocytes (Ueno, 1972) not manifested by equivalent concentrations of the parent compounds. The neutral $H_2O_2$-adducts such as histidine and histidyl histidine are strongly cytotoxic because they appear to enter cells in less than a minute. This is demonstrated by the fact that while the adducts are readily decomposed by catalase, albeit slower than $H_2O_2$ itself, addition of catalase to $H_2O_2$ or $H_2O_2$-histidine adduct prior to inoculation of a suspension of *Salmonella typhimurium* $LT_2$ removes the bacteriostatic action. Addition of catalase within 1 min after the inoculum has little effect on the activity of the adduct, but nearly eliminates that due to $H_2O_2$ alone (Schubert et al., 1969).

When oxygenated solutions of DNA or DNA constituents are irradiated, $H_2O_2$ is produced along with hydroperoxides. The latter result from the interaction of molecular oxygen with the free radicals produced by hydrogen abstraction on nucleic acid bases following OH attack (Ward, 1978). The major products, hydroxy hydroperoxides are unstable. Those of thymine have received much attention because of their suggested role in radiation-induced mutagenesis (Cerutti, 1974; Thomas et al., 1976; Wang et al., 1979).

Our goal in this investigation was to examine the disposition of low concentrations of $H_2O_2$ produced in irradiated, oxygenated solutions of nucleic acid constituents or added to non-irradiated solutions. In order to detect and evaluate $H_2O_2$-adduct formation in non-irradiated systems we utilized a sugar competition test in which we measured the rate of decomposition of $H_2O_2$ by glucose and/or fructose as modified by the test molecule (Schubert et al., 1969; Schubert, 1974). The rate of decomposition of $H_2O_2$ in irradiated solutions of nucleic acid constituents was determined by periodical analysis for $H_2O_2$ during storage at 4 $^{o}C$ under sterile conditions.

## MATERIALS AND METHODS

### *Chemicals*

Hydrogen peroxide, glucose, D-ribose, 2-deoxy-D-ribose, adenine, thymine and uracil were obtained from Merck (Darmstadt, West-Germany). Cytosine, adenosine, cytidine, thymidine and uridine were purchased from Aldrich Europe (Beerse, Belgium); 2'-deoxyadenosine, 2'-deoxycytidine and 2'-deoxyuridine from Sigma Chemical Company (St. Louis, MO, U.S.A.). Fructose was bought from BDH Chemicals Ltd. (Poole, England). All other chemicals were of the highest purity available.

*Glucose- and fructose-competition test*

Glucose and other sugars react at controllable rates with $H_2O_2$ in the absence of a catalyst (Moody, 1963; Moody, 1964). The decomposition of $H_2O_2$ is a function of temperature, concentration and pH. For example, at a concentration of $3.0 \times 10^{-4}$ M, $H_2O_2$ disappears from a phosphate-buffered (pH 7.0) glucose solution ($2.8 \times 10^{-3}$ M) at 37 °C with a half-life ($T_{\frac{1}{2}}$) of about 48 hours. In the presence of $2.9 \times 10^{-2}$ M histidine the $T_{\frac{1}{2}}$ of $H_2O_2$ is greater than 20 days. In the experiments described here we chose conditions which provided $T_{\frac{1}{2}}$'s optimum for our working conditions.

The test solutions consisted of 0.05 M glucose or fructose, 0.05 M phosphate buffer (pH 7.0), $6 \times 10^{-4}$ M $H_2O_2$ and 0.01 M test compound. Adenine and thymine were tested at different concentrations. In each experiment a corresponding control without test compound was run. All operations were and must be carried out under sterile conditions using sterilized glassware and filter sterilized solutions. The solutions were kept in brown stoppered bottles at 37 °C (waterbath). Samples were withdrawn periodically and analyzed for $H_2O_2$ by the titanium sulphate method of Egerton et al. (1954).

*Irradiation*

Solutions (0.01 M) of the nucleic acid constituents were prepared with 0.05 M phosphate buffer (pH 7.0). All solutions were irradiated at ambient temperature with a $^{60}Co$ gamma-source (Pilot Plant for Food Irradiation, Wageningen, The Netherlands) at a dose rate of 0.12-0.14 kGy/min. A total dose of 10 kGy was given. Before (30 min) and during irradiation, high purity oxygen was bubbled through the solutions. After irradiation, the solutions were stored at 4 °C under sterile conditions. Samples were periodically analyzed for $H_2O_2$ with the titanium sulphate method of Egerton et al. (1954).

## RESULTS

*Half-time of $H_2O_2$ in sugar media*

Fig. 1 shows the decomposition of $H_2O_2$ at 37 °C in a phosphate-buffered medium (pH 7.0) with and without sugar (glucose or fructose). Control peroxide solutions without added sugar were stable. The half-time ($T_{\frac{1}{2}}$) of $H_2O_2$ in the medium containing glucose is approximately 25 h. In fructose the $T_{\frac{1}{2}}$ is about 7.5 h. These results agree with those of Moody (1963) who showed that the rate of disappearance of $H_2O_2$ in fructose systems was 3-4 times higher than in glucose systems.

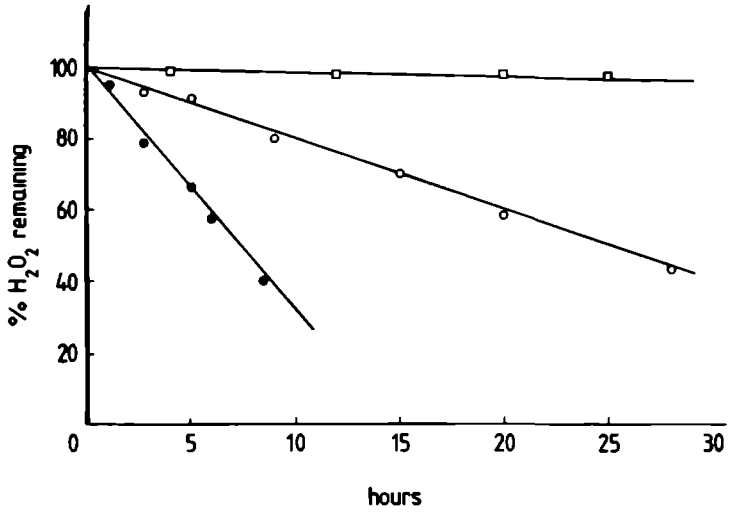


Figure 1. Decomposition of $H_2O_2$ ($6 \times 10^{-4}$ M) at 37 °C in phosphate-buffered (pH 7.0) solutions containing glucose (0.05 M) or fructose (0.05 M). □–□ control (no sugar); o–o glucose; ●–● fructose.

*Influence of metals and complexing agents*

Since adventitious amounts of trace metal ions, especially of free and complexed iron and copper, might be present in our test system, we investigated their influence on the decomposition of $H_2O_2$. It is well known that many of the complexed or chelated forms of Fe and Cu are capable of catalyzing the rate of $H_2O_2$ breakdown at neutral pH in phosphate buffer as, for example, the Cu-histidine chelate (Sharma and Schubert, 1971). Accordingly, we measured the effect of the disodium dihydrogen salt of EDTA alone and in the presence of $Fe^{2+}$, both in the absence and presence of fructose (Table 1). The inorganic salt $Fe(NH_4)_2(SO_4)_2$ at a concentration of $10^{-5}$ M had little effect on the rate of breakdown of $H_2O_2$ in the phosphate-buffered system containing 0.05 M fructose. However in the presence of excess EDTA, the decomposition of $H_2O_2$ was accelerated, and dependent on the concentration of the Fe-EDTA complex. EDTA by itself retards the reaction of $H_2O_2$ with fructose.

We also investigated the effect of histidine (known to form a stable adduct) in the presence and absence of $Fe^{2+}$, on $H_2O_2$ decomposition in the fructose system (Fig. 2). The results show that the decomposition of $H_2O_2$ is greatly retarded in fructose solutions containing 0.01 M histidine, while in solutions containing additional $Fe^{2+}$ ($10^{-5}$ M) $H_2O_2$ disappeared within 3 h. Similar results were also obtained with adenine and $Fe^{2+}$ in the fructose test.

Table 1. Influence of $Fe^{2+}$, EDTA and $Fe^{2+}$-EDTA on the decomposition of $H_2O_2$ ($6 \times 10^{-4}$ M) at 37 °C in phosphate-buffered solutions (pH 7.0) in the absence and presence of fructose (0.05 M).

| Fructose | EDTA (M) | $Fe^{2+}$ (M) | $T_{\frac{1}{2}}$ of $H_2O_2$ |
|---|---|---|---|
| - | - | - | > 3 weeks |
| - | $10^{-3}$ | - | 170.7 h |
| - | $10^{-2}$ | - | 37.1 h |
| - | - | $10^{-5}$ | > 3 weeks |
| - | - | $10^{-4}$ | n.d.[a] |
| - | $10^{-3}$ | $10^{-5}$ | 63.5 h |
| - | $10^{-3}$ | $10^{-4}$ | 11.3 h |
| - | $10^{-2}$ | $10^{-5}$ | 17.5 h |
| - | $10^{-2}$ | $10^{-4}$ | 2.6 h |
| + | - | - | 7.8 h |
| + | $10^{-3}$ | - | 33.7 h |
| + | $10^{-2}$ | - | 15.9 h |
| + | - | $10^{-5}$ | 6.6 h |
| + | - | $10^{-4}$ | n.d.[a] |
| + | $10^{-3}$ | $10^{-5}$ | 1.3 h |
| + | $10^{-3}$ | $10^{-4}$ | 0.2 h |
| + | $10^{-2}$ | $10^{-5}$ | 1.3 h |
| + | $10^{-2}$ | $10^{-4}$ | 0.2 h |

[a] not determined due to precipitation of $Fe_3(PO_4)_2$

The importance of the above findings is the fact that in the event transition metal ions are present in the sugar competition test for $H_2O_2$-adducts, the catalytic effect would be opposite to the usual retardation effect observed with molecules capable of forming polyfunctional hydrogen bonds, i.e. the observed effects were not due to artifacts (see next section).

*Competition tests with nucleic acid constituents*

We find it convenient to express the rate of breakdown of $H_2O_2$ in the presence of nucleic acid bases and nucleosides as:

$$k = \frac{T_{\frac{1}{2}} \ (H_2O_2) \text{ with sugar + test compound}}{T_{\frac{1}{2}} \ (H_2O_2) \text{ with sugar}}$$

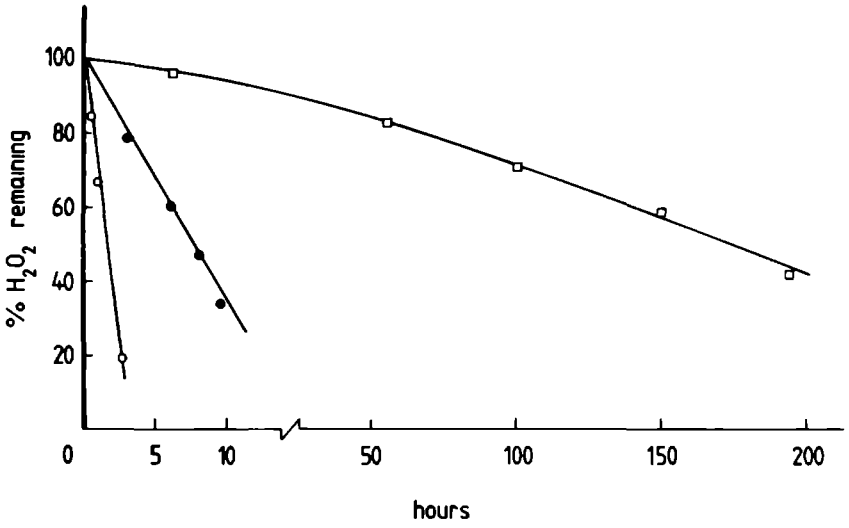


Figure 2. Fructose-competition test with histidine (0.01 M) in the absence and presence of $Fe^{2+}$ ($10^{-5}$ M). Initial concentration of $H_2O_2$: 6 x $10^{-4}$ M.
●—● control (only fructose); □—□ histidine (0.01 M); o—o histidine (0.01 M) + $Fe^{2+}$ ($10^{-5}$ M).

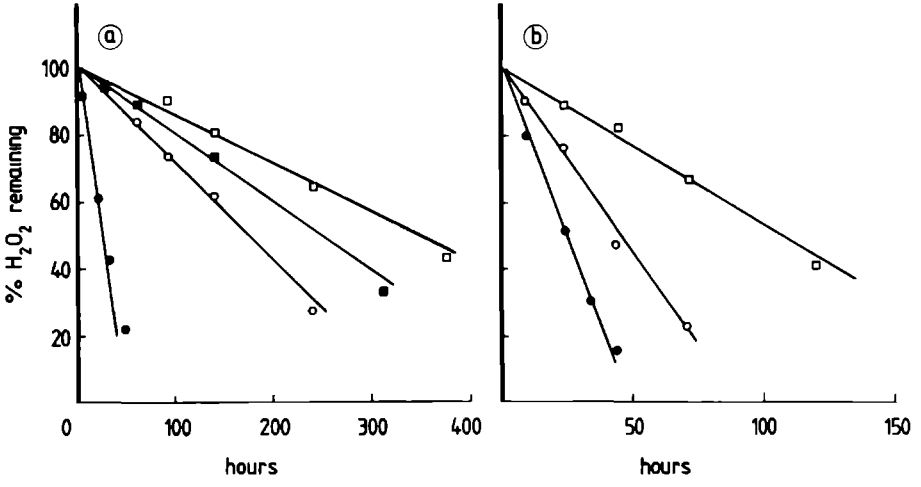


Figure 3. Glucose-competition test with different concentrations of adenine (a) and thymine (b). Initial concentration of $H_2O_2$: 6 x $10^{-4}$ M.
(a) ●—● control (only glucose); o—o 0.001 M adenine;
■—■ 0.005 M adenine; □—□ 0.01 M adenine
(b) ●—● control (only glucose); o—o 0.001 M thymine;
□—□ 0.01 M thymine.

Table 2. The interaction of $H_2O_2$ ($6 \times 10^{-4}$ M) with nucleic acid constituents (0.01 M) as measured with the glucose- and fructose-competition test.

| Compound | k-values[a,b] | |
|---|---|---|
| | in glucose | in fructose |
| adenine | 11.7 ± 1.9 | 6.4 ± 1.0 |
| thymine | 4.0 ± 0.8 | 1.8 ± 0.1 |
| cytosine | 2.2 ± 0.5 | 3.9 ± 0.3 |
| uracil | 1.6 ± 0.3 | 1.6 ± 0.3 |
| adenosine | 6.0 ± 0.1 | 4.0 ± 0.9 |
| thymidine | 3.0 ± 0.1 | 1.6 ± 0.4 |
| cytidine | 2.2 ± 0.6 | 2.7 ± 0.1 |
| uridine | 1.4 ± 0.6 | 1.5 ± 0.2 |
| D-ribose | - | 0.8 ± 0.1 |
| 2-deoxy-D-ribose | - | 0.8 ± 0.1 |

[a] For definition of k-value: see text
[b] Values are means (± S.D.) of at least two separate experiments

The k-values for the various compounds (0.01 M) tested are listed in Table 2. In both glucose- and fructose systems, adenine and adenosine are most effective in retarding the decomposition of $H_2O_2$, suggesting a strong interaction between $H_2O_2$ and adenine. The order of effectiveness of bases and nucleosides in glucose and fructose is similar except that the positions of thymine and cytosine are reversed. In glucose, we find: adenine > thymine > cytosine > uracil, while in fructose the order is: adenine > cytosine > thymine > uracil. The same phenomenon is observed with the nucleosides. Generally, the k-values are higher in glucose than in fructose, which probably reflects the faster reaction of $H_2O_2$ with fructose. With the nucleic acid bases we find higher k-values than with the nucleosides. This indicates that $H_2O_2$ also reacts with the sugar moiety of the nucleosides. Accordingly, we investigated D-ribose (0.01 M) and 2-deoxy-D-ribose (0.01 M), the sugar moieties of respectively ribonucleosides and deoxyribonucleosides in the fructose competition test. With both compounds, we find a k-value of 0.8 ± 0.1. Obviously, the sugar moieties contribute to the decomposition of $H_2O_2$ in fructose solutions.

The effect of varying the concentration of adenine and thymine was studied in the glucose test. The results presented in Fig. 3, show that the $T_{\frac{1}{2}}$ of $H_2O_2$ decreases with decreasing concentration of the nucleic acid base.

Table 3. $H_2O_2$ concentrations and $T_{\frac{1}{2}}$ of $H_2O_2$ decomposition in gamma-irradiated (10 kGy), oxygenated, buffered solutions (pH 7.0) solutions of nucleic acid constituents (0.01 M).

| Nucleic acid constituent | $H_2O_2$ (x $10^{-4}$ M)[a] | $T_{\frac{1}{2}}$ (days) |
|---|---|---|
| adenine | 19.7 | > 20 |
| thymine | 14.8 | 20 |
| cytosine | 15.7 | 8 |
| uracil | 20.4 | 11 |
| adenosine | 16.1 | > 25 |
| cytidine | 7.6 | 3 |
| uridine | 6.2 | 1 |
| 2'-deoxyadenosine | 16.2 | > 15 |
| thymidine | 8.7 | 7 |
| 2'-deoxycytidine | 10.3 | 6 |
| 2'-deoxyuridine | 7.6 | 4 |
| D-ribose | 13.4 | 1.5 |
| 2-deoxy-D-ribose | 15.6 | 4 |

[a] $H_2O_2$, determined immediately post-irradiation

*Irradiation experiments*

Table 3 shows the amounts of $H_2O_2$ determined immediately post-irradiation in oxygenated solutions of nucleic acid bases, ribo- and deoxyribonucleosides and sugar moieties and the half-time of $H_2O_2$ decomposition in these solutions. Although the peroxide concentrations are not the same and the conditions not fully comparable with those of the competition tests, adenine and (deoxy)adenosine are here also the most effective in retarding the decomposition of $H_2O_2$.

Considering the different conditions, these results are in reasonable agreement with those of the competition tests.

## DISCUSSION

By analogy with the known crystal structure of the histidine-peroxide adduct (Schubert, unpublished work) it is suggested that one structure of the adenine-$H_2O_2$ adduct is that postulated in Fig. 4. Adenine possesses, under neutral conditions, three potential hydrogen bonding acceptor sites and one donor site compared to two acceptor and one donor site for cytosine and thymine (Marsh, 1968). The pH plays an important role in hydrogen bonding. Thus, the ring nitrogen atom can switch from being an acceptor to a donor of the

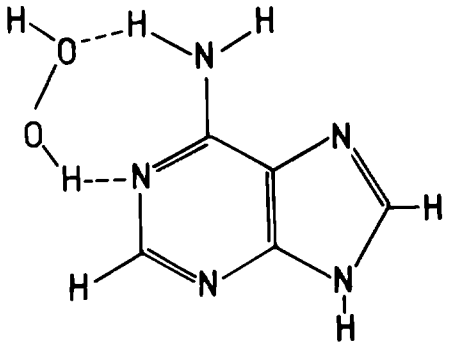

Figure 4. Postulated structure of an adenine-$H_2O_2$ adduct.

hydrogen. The particular sites involved will, in the case of the nucleic acid bases, depend on the tautomeric form. The capacity for polyfunctional hydrogen bonding giving cyclic or chelate structures permits these base-$H_2O_2$ adducts to remain intact in aqueous solution despite the fact that water reduces the free energy of association of hydrogen bonding compared to the solid state as was pointed out by Jencks (1969).

It is most likely that substituted (organic) peroxides also form adducts with polyfunctional molecules. However, the stability of the hydrogen-bonded structures is probably less than those formed with $H_2O_2$ since the bond energies of -O-O- in hydroperoxides, for example, are considerably less than in hydrogen peroxide (Curci and Edwards, 1970).

Since numerous polyfunctional structures, both simple and polymeric, are capable of hydrogen bonding and are present in biological systems, it is interesting to speculate that some of the so-called after effects in irradiated systems, e.g. biological and chemical such as viscosity effects on irradiated DNA (Conway, 1954; Alper, 1954; Schubert, 1969) are due to the peroxide adducts as has been cited for irradiated histidine solutions (Schubert et al., 1969). It is also interesting to speculate that part of the mutagenic and lethal effects of ionizing radiation on cells may be due to the formation of nuclear peroxides which by tending to form adducts may interfere with the normal base-pairing in DNA.

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# SUMMARY AND CONCLUSIONS

Deoxyribonucleic acid (DNA) is the most critical target for the lethal, mutagenic and carcinogenic effects of ionizing radiation on living organisms. Radiation-induced damage to DNA includes single-strand and double-strand breaks, modification and liberation of nucleic acid bases, alterations in the sugar moiety, and release of inorganic phosphate. Although many radiolytic products of DNA constituents have now been identified, it is largely unknown which changes in DNA are lethal, mutagenic or carcinogenic.

The aim of this study was to investigate the mutagenicity of gamma-irradiated solutions of nucleic acid constituents. For this purpose, two short-term mutagenicity test systems were used: the Salmonella/mammalian microsome test (Ames test) and the *in vitro* Sister-chromatid exchange test with Chinese hamster ovary cells. The results of this study are presented in five papers.

*Paper I*

Paper I deals with the mutagenicity of gamma-irradiated, oxygenated, buffered solutions of 2-deoxy-D-ribose (0.01 M), the sugar moiety of DNA. Solutions were irradiated with various doses and tested for mutagenicity, with and without pre-incubation, towards various strains of *Salmonella typhimurium*. The investigations revealed that irradiated solutions of 2-deoxy-D-ribose were mutagenic for TA 100 and TA 98, and non-mutagenic for TA 1535, TA 1537 and TA 1538. The mutagenic response increased with increasing radiation dose and with the quantity of irradiated sugar. The pre-incubation assay showed a higher response than the plate assay.

The use of S9 mix (metabolic activation system) in the Ames test or addition of catalase to the irradiated solutions (to destroy radiolytic hydrogen peroxide) had no effect on the mutagenic response. It was concluded that the observed mutagenicity was not due to hydrogen peroxide or malonaldehyde (MDA), both of which are among the products formed upon irradiation of oxygenated solutions of 2-deoxy-D-ribose. The radiation-induced mutagenicity of 2-deoxy-D-ribose solutions is most likely caused by carbonyl and/or dicarbonyl derivatives of this sugar.

*Paper II*

Paper II describes the mutagenicity of irradiated solutions of 2-deoxy-D-ribose under different experimental conditions, which may vary the yield and nature of the potentially mutagenic radiolytic products. Irradiation experiments were carried out in different media (water or buffer), in different atmospheres ($N_2$, $N_2O$, $O_2$) and with various concentrations of 2-deoxy-D-ribose. With irradiated (dose 10 kGy), buffered solutions of 2-deoxy-D-ribose (0.01 M) the mutagenicity towards TA 100, in both plate- and pre-incubation assay, showed the following relationship: $O_2 > N_2O > N_2$. With TA 98 (pre-incubation assay) the relationship was: $N_2O > N_2 > O_2$. These results indicate that the mutagenic effects are caused by one or more radiolytic products of 2-deoxy-D-ribose capable of inducing both base-pair and frameshift mutagenicity. The difference in order of mutagenicity for TA 100 and TA 98 might reflect the difference in yield and nature of the mutagenic products as a result of differences in the relative yields and nature of the primary radicals. Post-irradiation heating of the solutions resulted in a temperature-dependent reduction of the mutagenicity, probably due to dehydration of the mutagenic compound(s).

The studies with various concentrations of 2-deoxy-D-ribose, irradiated in the presence of oxygen, revealed a maximum mutagenic response of strain TA 100 at a concentration of 0.01 M. With TA 98 (pre-incubation assay) peak values occurred at both 0.01 and 0.05 M. The variations in mutagenicity with increasing concentrations of 2-deoxy-D-ribose are due to an increase in the degree of direct action of ionizing radiation, which may also influence the yield and nature of the mutagenic radiolytic products.

Irradiated non-buffered solutions of 2-deoxy-D-ribose (0.01 M) were generally less mutagenic than the corresponding buffered solutions. The order of mutagenicity for TA 100 was the same as found with the buffered solutions. With TA 98 no significant differences in the mutagenicity of irradiated $N_2$-, $N_2O$- or $O_2$-saturated solutions were observed.

At least in the pre-incubation assay (TA 100), the results of the mutagenicity studies with irradiated buffered and non-buffered solutions of 2-deoxy-D-ribose could be well correlated with the analyses for non-MDA aldehydes. However, no definite conclusions could be drawn on the product(s) specifically involved.

With D-ribose, the corresponding sugar moiety in RNA, the highest mutagenicity for both strains was found with irradiated (dose 10 kGy), $N_2O$- and $N_2$-saturated solutions (0.01 M). Oxygenated solutions did not show appreciable mu-

tagenicity. Irradiated solutions of D-ribose were considerably less mutagenic than those of 2-deoxy-D-ribose.

*Paper III*

This paper describes the results of the mutagenicity studies (Ames test) with irradiated (dose 10 kGy) $N_2$-, $N_2O$- and $O_2$-saturated buffered solutions of nucleic acid bases, nucleosides and a nucleotide (0.01 M). The irradiated solutions of nucleic acid bases were not mutagenic for TA 100 and TA 98, in both plate- and pre-incubation assay. With the exception of oxygenated solutions of thymidine, which roughly doubled the spontaneous mutation rate of TA 100, no appreciable mutagenicity towards this strain (in the plate assay) was found with irradiated solutions of nucleosides. Using the more sensitive pre-incubation assay with TA 100, irradiated oxygenated solutions of thymidine and 2'-deoxyuridine were found to be weakly mutagenic. $N_2O$- and $N_2$-saturated solutions of all four nucleosides tested were clearly mutagenic for TA 100 in this assay. $N_2O$-saturated solutions were generally more mutagenic than the corresponding $N_2$-saturated solutions.

These results indicate that the OH radicals are mainly responsible for the formation of mutagenic radiolytic products. The mutagenicity of irradiated solutions of nucleosides and 2-deoxy-D-ribose (shown in papers I and II) and the non-mutagenicity of irradiated solutions of nucleic acid bases led to the conclusion that the sugar moiety of the nucleosides is the main target from which the radicals produce mutagenic products.

In view of the preceding results, it appeared worthwhile to study the possible mutagenicity of irradiated solutions of nucleotides, in which the sugar molecule is covalently bound to a nucleic acid base as well as to a phosphate group. Irradiated $N_2$-, $N_2O$- and $O_2$-saturated solutions of thymidine-5'-monophosphate (TMP) did not show mutagenicity for TA 100 in both plate- and pre-incubation assay. In this respect it is interesting to note that radiolytic sugar products - suggested to be mutagenic - have not been found in irradiated solutions of TMP. The chemical constitution of the sugar moiety in nucleotides apparently prevents the formation of such (mutagenic) products.

*Paper IV*

Induction of sister-chromatid exchanges (SCE s) and chromosomal aberrations in Chinese hamster ovary (CHO) cells by hydrogen peroxide and gamma-irradiated (dose 10 kGy) oxygenated buffered and non-buffered solutions of solutions of

2-deoxy-D-ribose, thymine and thymidine was studied by using a modified BrdUrd-labelling method. A slight increase in frequencies of SCE s was observed with hydrogen peroxide up to a concentration of $10^{-3}$ M. Irradiated solutions of 2-deoxy-D-ribose clearly induced sister-chromatid exchanges in CHO cells. The increase was linear with the concentration of 2-deoxy-D-ribose. Non-buffered solutions of 2-deoxy-D-ribose were more effective in inducing SCE s than buffered solutions. These results confirm the findings of the Salmonella test that irradiated solutions of 2-deoxy-D-ribose are mutagenic. The SCE test also shows that hydrogen peroxide can be ruled out as a mutagenic agent in irradiated solutions of 2-deoxy-D-ribose.

The frequencies of SCE s induced by irradiated oxygenated solutions of thymine and thymidine were slightly higher than the control values. No linear relationship with concentration was found. Non-buffered solutions were here again more effective than buffered solutions. The results of the SCE test with these compounds are not fully comparable with those of the Salmonella test.

The frequencies of chromosomal aberrations were enhanced by hydrogen peroxide at a concentration of $10^{-2}$ M which is a factor 5-7 above the hydrogen peroxide concentrations found in the irradiated solutions of 2-deoxy-D-ribose. Irradiated non-buffered solutions of 2-deoxy-D-ribose were very effective in inducing (iso) chromatid breaks and chromatid exchanges. The induction of both types of aberrations were concentration dependent. Irradiated buffered solutions of 2-deoxy-D-ribose were considerably less effective in inducing chromosomal aberrations.

The frequencies of chromosomal aberrations were slightly enhanced by irradiated solutions of thymine and thymidine. The results did not indicate a clear-cut relationship with the concentrations used. Irradiated non-buffered solutions of both thymine and thymidine were more effective than the corresponding buffered solutions.

*Paper V*

Here the fate of low concentrations of $H_2O_2$ (produced radiolytically or added chemically) in buffered solutions of nucleic acid bases and nucleosides (0.01 M) is studied. In order to detect hydrogen peroxide adduct formation in non-irradiated solutions of nucleic acid constituents a sugar competition test was used to measure the rate of decomposition of hydrogen peroxide by glucose and/or fructose as modified by the test molecule. In both glucose- and fructose systems, adenine and adenosine are most effective in retarding the decom-

position of $H_2O_2$, suggesting a strong interaction (via hydrogen bonding) between adenine and $H_2O_2$.

The influence of low concentrations of free and complexed $Fe^{2+}$ on the rate of decomposition of $H_2O_2$ was studied in phosphate buffered solutions in the absence and presence of fructose. These investigations revealed that $Fe^{2+}$ at a concentration of $10^{-5}$ M had little or no effect on the breakdown of $H_2O_2$ in these systems. In the presence of excess EDTA, the decomposition of $H_2O_2$ was accelerated, and dependent on the concentration of the Fe-EDTA complex.

The half-time of $H_2O_2$ decomposition in irradiated (dose 10 kGy) oxygenated buffered solutions of nucleic acid constituents was determined by periodical analysis for $H_2O_2$ during storage at 4 $^{o}$C. Adenine and adenosine were here also the most effective in retarding the decomposition of $H_2O_2$, which confirmed the results of the sugar competition test.

The major conclusions of the investigations, presented in this thesis, are:

1. Irradiated solutions of 2-deoxy-D-ribose, the sugar moiety in DNA, are clearly mutagenic in *Salmonella typhimurium* and induce sister-chromatid exchanges in CHO cells. The mutagenicity of these solutions is not due to hydrogen peroxide or malonaldehyde.
2. Irradiated solutions of nucleic acid bases do not show mutagenic activity in the Salmonella test.
3. Irradiated solutions of nucleosides are mutagenic in *Salmonella typhimurium*. The mutagenicity is due to the radiolytic products of the sugar moiety.
4. Irradiated solutions of the nucleotide, thymidine-5'-monophosphate, are not mutagenic in *Salmonella typhimurium*.

In addition to these conclusions, the following remarks can be made. (1) 5'-TMP does not probably represent the other nucleotides. From the literature it is known, for example, that irradiated 3'-monophosphate-nucleotides and 3',5'-diphosphate-nucleotides generally eliminate more readily the phosphate groups than irradiated 5'-nucleotides. Subsequently, mutagenic radiolytic products might arise from the sugar moiety of the resulting nucleosides. (2) Upon irradiation of DNA an identical situation may arise. Very recent investigations of Schulte-Frohlinde (Proceedings of the Sixth International Congress of Radiation Research, Tokyo, 1979) show that in irradiated solutions of DNA products are formed - initially by breakage of phosphate ester bonds or by a C-C bond rupture in the sugar skeleton - which are identical to the radiolytic products of 2-deoxy-D-ribose.

It is difficult to extrapolate the presented results and considerations to irradiated foods. In principal, it seems possible that radiolytic sugar products of DNA are formed in irradiated foods. It can be assumed that, at the doses applied in food irradiation processes e.g. to inhibit sprouting of potatoes or onions, the concentrations of these products will be very low and/or that they react with other components in foods, in such a way that they become harmless. The results of the investigations on the wholesomeness of irradiated foods, which never showed any deleterious effect, seem to justify this assumption.

# SAMENVATTING EN CONCLUSIES

De lethale, mutagene en carcinogene effecten van ioniserende straling op levende organismen zijn het gevolg van schade aan desoxyribonucleïnezuur (DNA). Ioniserende straling beschadigt DNA op verschillende manieren, onder andere door modificatie en afsplitsing van nucleïnezuur basen, veranderingen in het suikermolecuul, en door afsplitsing van de fosfaatgroep. Hoewel veel radiolyseprodukten van de verschillende DNA componenten geïdentificeerd zijn, is het tot nu toe grotendeels onbekend welke veranderingen in DNA lethaal, mutageen of carcinogeen zijn.

Het doel van deze studie was de mutageniteit te onderzoeken van bestraalde oplossingen van nucleïnezuur componenten. Hiertoe werden twee kortdurende mutageniteitstesten gebruikt: de Salmonella/microsoom test (Ames test) en de *in vitro* Sister-chromatid exchange test met ovarium cellen van de Chinese hamster. De resultaten van deze studie werden gepresenteerd in een 5-tal publikaties.

*Publikatie I*

Publikatie I behandelt de mutageniteit van bestraalde, met zuurstof verzadigde, gebufferde oplossingen van 2-desoxy-D-ribose (0.01 M), het suikermolecuul van DNA. De oplossingen werden bestraald met verschillende doses en onderzocht op mutageniteit, met en zonder pre-incubatie, in verschillende stammen van *Salmonella typhimurium*. Uit dit onderzoek blijkt dat bestraalde oplossingen van 2-desoxy-D-ribose mutageen zijn in de stammen TA 100 en TA 98, en niet mutageen in TA 1535, TA 1537 en TA 1538. De mutageniteit nam toe met toenemende stralingsdosis en met de hoeveelheid van de bestraalde suikeroplossing. De mutagene effecten waren groter in de pre-incubatietest dan in de plaattest. Het gebruik van S9 (metabool activerings systeem) in de Ames test of het toevoegen van catalase aan de bestraalde oplossingen (met het doel waterstofperoxide te vernietigen) had géén invloed op de mutageniteit. Uit het onderzoek blijkt verder dat de mutageniteit niet wordt veroorzaakt door waterstofperoxide of malonaldehyde (MDA). Beide produkten worden, naast een groot aantal andere, gevormd in bestraalde oplossingen van 2-desoxy-D-ribose. Het is meer waarschijnlijk dat de door straling geïnduceerde mutageniteit wordt veroorzaakt door carbonyl en/of dicarbonyl derivaten van deze suiker.

*Publikatie II*

Publikatie II beschrijft de mutageniteit van 2-desoxy-D-ribose, bestraald onder verschillende experimentele condities, waardoor de aard en hoeveelheid van mogelijk mutagene radiolyseprodukten kan worden beïnvloed. De bestralingen werden uitgevoerd in verschillende media (water of buffer), onder doorleiden van verschillende gassen ($N_2$, $N_2O$, $O_2$) en met verschillende concentraties van 2-desoxy-D-ribose. Met bestraalde (dosis 10 kGy), gebufferde oplossingen van 2-desoxy-D-ribose werd de volgende relatie tussen de mutageniteit in TA 100 (zowel in de plaat- als in de pre-incubatietest) en de verschillende gascondities gevonden: $O_2 > N_2O > N_2$. In TA 98 (in de pre-incubatietest) was deze relatie: $N_2O > N_2 > O_2$. De resultaten tonen aan dat de mutagene effecten veroorzaakt worden door één of meerdere radiolyseprodukten van 2-desoxy-D-ribose die in staat zijn om zowel basepaar als frameshift mutaties te induceren. Het verschil dat gevonden werd tussen de stammen TA 100 en TA 98 betreffende de relatie tussen mutageniteit en gascondities zou een afspiegeling kunnen zijn van het verschil in de hoeveelheid en aard van mutagene produkten dat mogelijk veroorzaakt wordt door verschil in de relatieve hoeveelheden en aard van de primaire radicalen. Het verhitten van bestraalde oplossingen resulteerde in een temperatuur-afhankelijke reductie van de mutageniteit, waarschijnlijk als gevolg van dehydratatie van mutagene produkten.

In het onderzoek met verschillende concentraties van 2-desoxy-D-ribose, bestraald in aanwezigheid van zuurstof, werd een maximaal mutageen effect in TA 100 gevonden met een concentratie van 0.01 M. In TA 98 (pre-incubatietest) werden maximale effecten waargenomen met zowel 0.01 als 0.05 M. De variaties in mutageniteit met toenemende concentraties van 2-desoxy-D-ribose zijn een gevolg van het feit dat de directe effecten van ioniserende straling bij hogere concentraties toenemen, hetgeen van invloed kan zijn op de aard en hoeveelheid van mutagene radiolyseprodukten.

Bestraalde, niet-gebufferde oplossingen van 2-desoxy-D-ribose (0.01 M) waren over het algemeen minder mutageen dan de overeenkomstige gebufferde oplossingen. De relatie tussen mutageniteit in TA 100 en de gascondities was dezelfde als werd gevonden met gebufferde oplossingen. In TA 98 werden geen significante verschillen in mutageniteit tussen bestraalde, met $N_2$-, $N_2O$- of $O_2$-verzadigde oplossingen aangetoond.

Met name in de pre-incubatietest (TA 100) liep de mutageniteit van de, onder verschillende omstandigheden, bestraalde oplossingen van 2-desoxy-D-ribose parallel met de concentraties van "niet-MDA" aldehyden in die oplossingen. De-

finitieve uitspraken ten aanzien van de mutageniteit van individuele radiolyseprodukten konden echter niet worden gedaan.

Bestraalde (dosis 10 kGy) oplossingen van D-ribose (0.01 M), het suikermolecuul van RNA, waren aanzienlijk minder mutageen dan die van 2-desoxy-D-ribose. Oplossingen van D-ribose, bestraald in aanwezigheid van $N_2O$ of $N_2$, waren mutageen in TA 100 en TA 98. Met oplossingen, bestraald in aanwezigheid van $O_2$ werden geen mutagene effecten gevonden.

*Publikatie III*

Publikatie III is gewijd aan de mutageniteit van bestraalde (dosis 10 kGy) oplossingen van nucleïnezuur basen, nucleosiden en een nucleotide (0.01 M). Bestraalde oplossingen van nucleïnezuur basen waren, zowel in de plaat- als in de pre-incubatietest, niet mutageen in *S. typhimurium* TA 100 en TA 98. Met uitzondering van de met zuurstof verzadigde oplossingen van thymidine, die de spontane mutatiefrequentie van TA 100 verdubbelden, werden er, in de plaattest geen mutagene effecten gevonden met bestraalde oplossingen van nucleosiden. In de meer gevoelige pre-incubatietest waren bestraalde, met zuurstof verzadigde oplossingen van thymidine en 2'-deoxyuridine zwak mutageen in TA 100. In deze test waren de met $N_2O$- en $N_2$-verzadigde oplossingen van alle geteste nucleosiden echter duidelijk mutageen. De met $N_2O$ verzadigde oplossingen waren daarbij over het algemeen meer mutageen dan de overeenkomstige, met $N_2$ verzadigde oplossingen.

Deze resultaten tonen aan dat voornamelijk de OH radicalen verantwoordelijk zijn voor de vorming van mutagene radiolyseprodukten. Uit het feit dat bestraalde oplossingen van nucleosiden en 2-desoxy-D-ribose mutageen, en bestraalde oplossingen van nucleïnezuur basen niet-mutageen zijn, kan worden geconcludeerd dat de mutagene produkten ontstaan als gevolg van stralingsschade in het suikergedeelte van de nucleosiden.

Gezien deze resultaten leek het van belang de mutageniteit van bestraalde oplossingen van nucleotiden te onderzoeken. In nucleotiden is het suikermolecuul covalent gebonden aan zowel een nucleïnezuur base als aan een fosfaatgroep. Oplossingen van thymidine-5'-monofosfaat (TMP) werden bestraald onder verschillende omstandigheden en getest op mutageniteit in TA 100. Zowel in de plaat- als in de pre-incubatietest werden geen mutagene effecten gevonden. In dit verband is het interessant op te merken dat radiolyseprodukten van de suiker niet zijn gevonden in bestraalde oplossingen van TMP. De binding van 2-desoxy-D-ribose aan nucleïnezuur base en fosfaatgroep verhindert blijkbaar de

vorming van dergelijke (mutagene) produkten.

*Publikatie IV*

In publikatie IV wordt de inductie van "sister-chromatid exchanges" (SCE's) en chromosoomafwijkingen in ovarium cellen van de Chinese hamster (CHO) door waterstofperoxide en gebufferde en niet-gebufferde oplossingen van 2-desoxy-D-ribose, thymine en thymidine, bestraald (dosis 10 kGy) in aanwezigheid van zuurstof, beschreven. Een geringe toename in de frequenties van SCE's werd waargenomen met waterstofperoxide tot en met een concentratie van $10^{-3}$ M. Bestraalde oplossingen van 2-desoxy-D-ribose induceerde duidelijk SCE's in CHO cellen. De toename in frequenties toonde een lineair verband met de concentratie. Niet-gebufferde oplossingen van 2-deoxy-D-ribose waren effectiever in het induceren van SCE's dan gebufferde oplossingen. Deze resultaten bevestigen de bevindingen van de Salmonella test, nl. dat bestraalde oplossingen van 2-desoxy-D-ribose mutageen zijn. De SCE test toont ook aan dat de mutageniteit van bestraalde oplossingen van 2-desoxy-D-ribose niet wordt veroorzaakt door waterstofperoxide.

De frequenties van SCE's, geïnduceerd door bestraalde oplossingen van thymine en thymidine waren iets hoger dan die van de controles. Het verband met de concentratie was ook niet lineair. Niet-gebufferde oplossingen waren ook hier effectiever dan gebufferde oplossingen. De resultaten van de SCE test met deze componenten zijn niet volledig vergelijkbaar met die van de Salmonella test.

De frequenties van chromosoomafwijkingen werden verhoogd door waterstofperoxide bij een concentratie van $10^{-2}$ M, hetgeen een factor 5 tot 7 hoger is dan de waterstofperoxide concentraties van bestraalde oplossingen van 2-desoxy-D-ribose. Bestraalde niet-gebufferde oplossingen van 2-desoxy-D-ribose induceerden zeer duidelijk (iso) chromatide breuken en "chromatid exchanges". De inductie van beide typen chromosoomafwijkingen was afhankelijk van de concentratie. Gebufferde oplossingen van 2-desoxy-D-ribose waren aanzienlijk minder effectief in het induceren van chromosoomafwijkingen.

De frequenties van chromosoomafwijkingen werden in geringe mate verhoogd door bestraalde oplossingen van thymine en thymidine. Er werd geen duidelijk verband met de gebruikte concentraties gevonden. Bestraalde niet-gebufferde oplossingen van zowel thymine als thymidine waren effectiever in het induceren van chromosoomafwijkingen dan de overeenkomstige, gebufferde oplossingen.

*Publikatie V*

De laatste publikatie beschrijft de interactie van waterstofperoxide met nucleïnezuur basen en nucleosiden, in bestraalde en niet-bestraalde oplossingen. De vorming van waterstofperoxide adducten in niet-bestraalde oplossingen van nucleïnezuur componenten werd bestudeerd met behulp van een zogenaamde competitietest. Hierin werd de invloed van nucleïnezuur componenten op de afbraaksnelheid van waterstofperoxide door glucose of fructose bepaald. In zowel de glucose- als fructose competitietest werd gevonden dat adenine en adenosine zeer effectief de afbraak van waterstofperoxide vertragen, hetgeen een sterke interactie (via vorming van waterstofbruggen) tussen adenine en waterstofperoxide suggereert.

De invloed van lage concentraties van vrije en gecomplexeerde ijzer (II) ionen op de afbraaksnelheid van waterstofperoxide werd bestudeerd in fosfaatbuffer, zowel in de afwezigheid als aanwezigheid van fructose. Dit onderzoek toonde aan dat vrije ijzer (II) ionen (concentratie $10^{-5}$ M) weinig of geen invloed hebben op de afbraak van waterstofperoxide. In aanwezigheid van een overmaat EDTA werd de afbraak van $H_2O_2$ sterk versneld. De reactiesnelheid was afhankelijk van de concentratie van het ijzer-EDTA complex.

De halfwaarde-tijd van waterstofperoxide in gebufferde oplossingen van de nucleïnezuur componenten, bestraald (dosis 10 kGy) in aanwezigheid van zuurstof) werd bepaald d.m.v. periodieke analyses van de waterstofperoxide concentratie gedurende bewaring bij 4 $^{o}$C. Adenine en adenosine waren relatief zeer effectief in het vertragen van de afbraak van waterstofperoxide, hetgeen de resultaten van de competitietest, ondanks verschil in condities, bevestigde.

De belangrijkste conclusies uit het onderzoek, gepresenteerd in dit proefschrift, zijn:

1. Bestraalde oplossingen van 2-desoxy-D-ribose, de suikercomponent van DNA, zijn duidelijk mutageen in *Salmonella typhimurium* en induceren sister-chromatid exchanges in CHO cellen. De mutageniteit van deze oplossingen wordt niet veroorzaakt door waterstofperoxide of malonaldehyde.
2. Bestraalde oplossingen van nucleïnezuur basen vertonen geen mutagene activiteit in de Salmonella test.
3. Bestraalde oplossingen van nucleosiden zijn mutageen in *Salmonella typhimurium*. De mutageniteit wordt veroorzaakt door de radiolyseprodukten van het suikermolecuul.
4. Bestraalde oplossingen van het nucleotide, thymidine-5'-monofosfaat, zijn niet mutageen in *Salmonella typhimurium*.

Bij deze conclusies kunnen nog de volgende kanttekeningen worden geplaatst. (1) 5'-TMP is waarschijnlijk niet representatief voor de andere nucleotiden. Uit de literatuur is het bijvoorbeeld bekend dat bestraalde 3'-monofosfaatnucleotiden en 3',5'-difosfaatnucleotiden veel gemakkelijker de fosfaatgroep(en) elimineren dan bestraalde 5'-nucleotiden. Uit het suikermolecuul van de resulterende nucleosiden zouden vervolgens mutagene radiolyseprodukten kunnen ontstaan. (2) Een zelfde situatie kan ontstaan bij bestraling van DNA. Zeer recente onderzoekingen van Schulte-Frohlinde (Verslag van het 6$^{e}$ Internationale Congres over Stralingsonderzoek, Tokyo, 1979) tonen aan dat in bestraalde oplossingen van DNA produkten ontstaan - in eerste instantie via verbreking van de fosfaat-ester banden of door verbreking van een C-C band in het suikergedeelte - die identiek zijn aan de radiolyseprodukten van 2-desoxy-D-ribose.

Extrapolatie van de gepresenteerde resultaten en overwegingen naar bestraald voedsel blijft een moeilijke zaak. In principe lijkt het mogelijk dat radiolyseprodukten uit het suikergedeelte van DNA in bestraald voedsel gevormd worden. Het is echter aannemelijk dat, bij de doses die in het voedselbestralingsprocédé toegepast worden om b.v. spruiten van aardappels of uien tegen te gaan, de concentraties van dergelijke produkten zeer laag zullen zijn en/of dat ze zodanig reageren met andere componenten van het voedsel dat ze onschadelijk worden. De resultaten van het onderzoek naar de "wholesomeness" van bestraald voedsel, dat nooit een schadelijk effect heeft aangetoond, lijken deze veronderstelling te rechtvaardigen.

## CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 7 februari 1951 te Delft en behaalde in 1969 het einddiploma Gymnasium B aan het St. Stanislascollege te Delft. In hetzelfde jaar begon hij met de studie Scheikunde aan de Katholieke Universiteit te Nijmegen. Het kandidaatsexamen ($S_2$) werd afgelegd in september 1972. Het doctoraalexamen Scheikunde met de hoofdvakken Organische Chemie en Farmacochemie, en als uitbreiding Quantumchemie, legde hij af in mei 1976. Gedurende de nakandidaatsperiode vervulde hij verschillende student-assistentschappen en was hij van augustus 1975 tot februari 1976 verbonden als leraar Natuurkunde aan de Scholengemeenschap Nijmegen-West te Nijmegen. Van 1 juli 1976 tot 1 januari 1980 was hij in dienst van de Stichting ITAL te Wageningen, alwaar het onderzoek beschreven in dit proefschrift grotendeels werd verricht.

# STELLINGEN

## I

De conclusie van Wang e.a. dat *cis*-5,6-dihydro-6-hydroperoxy-5-hydroxy-thymine (6-TOOH) sterk mutageen is in *Salmonella typhimurium* (Ames test) is, op grond van de gepresenteerde resultaten, onjuist.

S.Y. Wang, B.S. Hahn, R.B. Batzinger en E.B. Bueding (1979)
Biochem. Biophys. Res. Commun., 89, 259-263.

Dit proefschrift

## II

Het is zeer aannemelijk dat de positieve resultaten van Minnich e.a. met 5-fluoruracil in de Ames test het gevolg zijn van het scoren van zogenaamde "pitpoints".

V. Minnich, M.E. Smith, D. Thompson en S. Kornfeld (1976)
Cancer, 38, 1253-1258.

Y. Seino, M. Nagao, T. Yahagi, A. Hoshi, T. Kawachi en T. Sugimura (1978)
Cancer Res., 38, 2148-2156.

## III

Nagao e.a. tonen onvoldoende aan dat de mutageniteit van koffie en thee in de Ames test niet wordt veroorzaakt door de aanwezigheid van histidine in de extracten.

M. Nagao, Y. Takahashi, H. Yamanaka en T. Sugimura (1979)
Mutation Res., 68, 101-106.

## IV

Bij het vaststellen van normen voor blootstelling aan chemische stoffen dient de mogelijk synergistische werking van de betrokken stoffen met andere agentia mede in overweging genomen te worden.

Alvorens afwijkende eigenschappen van *in vitro* gekweekte plantecellen toe te schrijven aan geïnduceerde mutaties, dient de cellulaire gevoeligheid voor het toegepaste mutagene agens te worden vastgesteld.

C.M. Colijn, A.J. Kool en H.J.J. Nijkamp (1979) Theor. Appl. Genet., 55, 101-106.

P.A.Th.J. Werry en K.M. Stoffelsen (1980) in Plant Cell Cultures; Results and Perspectives (Uitg. F. Sala, B. Parisi, R. Cella en O. Cifferi), Elsevier, Amsterdam, pp. 115-120.

VI

Bij de bestudering van de door straling geïnduceerde inactivering van enzymen en microörganismen via het selectieve radicaalanion, $Br_2^{-\cdot}$, wordt dikwijls onvoldoende rekening gehouden met mogelijke effecten van het medium.

D. Becker, J.L. Redpath en L.I. Grossweiner (1978) Radiat. Res., 73, 51-74.

VII

De door Schmidt en Raftery gevolgde methode ter zuivering van de acetylcholine receptor uit de electroplaxmembranen van *Torpedo californica* lijkt eerder te berusten op ionenuitwisselings- dan op affiniteitschromatografie.

J. Schmidt en M.A. Raftery (1973) Biochemistry, 12, 852-856.

VIII

Het schijnt de meeste Nederlandse sportjournalisten te ontgaan dat van een klein land niet kan worden geëist dat alle overwinningen en wereldrecords door Nederlandse mannen en vrouwen worden behaald.

Nijmegen, 26 september 1980. J.W.G.M. WILMER

DRUK PUDOC
WAGENINGEN